Universidade de Lisboa
Faculdade de Medicina de Lisboa

PHENOTYPES IN INFLAMMATORY BOWEL DISEASE

Joana Maria Tinoco da Silva Torres

Orientador(es):

Prof. Doutora Marília Lopes Cravo

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Tese especialmente elaborada para obtenção do grau de Doutor no Ramo de Medicina, especialidade de Gastrenterologia.

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- Doutora Marília Lopes Cravo, Professora Associada Convidada com Agregação da Faculdade de Medicina da Universidade de Lisboa (Orientadora).

2018

PROLOGUE AND ACKOWLEDGMENTS

As I began my gastroenterologist training I held on to my long-time desire to dedicate my efforts to both clinical and translational research. In 2009, I had the opportunity to spend 3 months in the Gastroenterology Department in Lille, France, where I met Professor Jean-Fréderic Colombel. At the time of my stay, the Gastroenterology Department in Lille was immersed with a multitude of research projects, from clinical trials, to investigator-initiated studies. Having the opportunity to see how the research was conducted and all the interaction between investigators and clinicians was stimulating and mind-opening. Therefore, despite being a short stay, this period was decisive to foster and blossom my interest in research. It was then in 2011, that the opportunity to start performing research appeared, when Professor Jean-Frédéric Colombel invited me to join him in his sabbatical year at Mount Sinai Hospital (MSH) in New York, working on translational research projects. By coincidence I had just finished my Gastroenterology training so the timing was perfect, and I spent one year in New York. I still remember when I arrived at Mount Sinai, and Professor Colombel told me we needed to start developing a protocol in the field of Primary Sclerosing Cholangitis (PSC) associated with Inflammatory Bowel Disease (IBD), a very rare but distinctive form of IBD. I never had written a protocol before and I must confess I was a little scared, but Professor Colombel, in a seamless way, helped and guided me, always keeping a working environment of dialogue and collaboration, and always challenging me to think out of the box. During this year, I also met Professor Steven H. Itzkowitz and Professor Jianzhong Hu, and alongside with Professor Colombel, we started to work on several projects in the field of PSC-IBD. This year went by fast, but it laid the ground and foundation for the work that followed. After 2011, I went back to Portugal, and spent three years dedicating most of my time to working in the IBD clinic, but we kept collaborating and developing research projects in the field of PSC-IBD. One of the most crucial events in my return to Portugal was the fact and luck that I started to work with Professor Marília Cravo, the Director of the Gastroenterology Department at Hospital Beatriz Ângelo, Portugal. She always trusted in my research and always motivated me to continue and pursue further avenues. She strongly supported me to go beyond clinical work and she provided me the time and means to pursue clinical research, and strongly supported my return to the United States, that happened following the invitation from Professor Jean-Frédéric Colombel, who had in the meanwhile become the Director of The IBD Center at MSH, New York. So, in January 2015, I returned to MSH, to pursue and develop further in IBD research. My goal in going back to the United

States was to become fully immersed in translational and clinical research related to Inflammatory Bowel Disease (IBD), with a special focus in the microbiome in specific settings, namely in PSC-IBD but also in the area of preclinical and early IBD. During the time spent at MSH, I worked on large cohort projects devoted to better understanding the preclinical changes that take place before an established IBD diagnosis, and more specifically studying the role of microbiota as causative for disease, with the overall aim of disease prevention. I was involved in several large projects, recruiting a vast number of patients and dealing with large volume of samples. I also resumed the ongoing projects in PSC-IBD, I brought in the projects I had started in Portugal and started new collaborations in the PSC-IBD topic. To compile all these projects and work, and to apply to a PhD was the next natural step, and therefore in 2015, motivated by Professor Marília Cravo, I signed up in the PhD program at the Centro Académico de Medicina de Lisboa.

The work I developed during these years is presented in this thesis, and it was performed mostly in Lisbon (both at Hospital Beatriz Ângelo and Professor Cecília Rodigues´lab) and New York (at Professor Steven H. Itzkowitz and Professor Jianzhong Hu´s labs), also counting with some collaborations from Professor Dominique Rainteau in Paris, and investigators in the Netherlands. I am truly thankful to all them for their generosity and for always welcoming my ideas and helping me to pursue my projects.

This thesis is divided in 5 chapters and a unifying conclusion. The first chapter is an introduction about inflammatory bowel disease and primary sclerosing cholangitis. This chapter results from the adaptation of several review articles and book chapters I had the opportunity to write over this time. The 2nd to the 4th chapters present the results of the work developed and the papers that were generated and published in different journals. Finally, I discuss how the work developed contributed to a better knowledge about the phenotype of PSC-IBD, as well as future avenues for research.

Finally, I would like to acknowledge and thanks all the people I got to work with and that facilitated and made possible the work here presented. I would like to thank my division co-workers that took on my clinical duties while I was away and that provide daily support for all the practical aspects of performing research. I would also like to thank the clinical director of my Hospital, Professor Rui Maio, for allowing me to go away for two years and providing

me the formal support from my Institution, as well my Hospital´s board of administration (Dr. Artur Vaz, Dr. Francisco Mota) and Engenheira Isabel Vaz, for having supported and keeping on supporting me, and my research and providing me the time to pursue it. Many thanks to Carolina Palmela for helping to recruit patients and samples; Xiuliang Bao, Ruiqui Huang, Shailja Sha, Joren tenHove, Daniel Castaneda, Anli Cheng, Alina Iuga, Noam Harpaz, Ana Paula Krieger and Professor David Sachar, for helping me in handling and processing of samples, collection of clinical data and/or analysis and interpretation of data. A special thanks to Professor Cecília Rodrigues for her availability and help in conducting the work presented in chapter 4 and to Professor Dominique Rainteau for helping to finalize the project. I would also like to thank Professor Steven H. Itzkowitz for his generosity and companionship during this time and for facilitating many of the projects that are presented in this Doctoral thesis. I would like to acknowledge and thanks Professor Jianzhong Hu who was crucial throughout these years and helped me to develop and work in these projects, and contributed immensely to my knowledge and skills in the field of microbiome, both in the lab as well in the analytical side. I also would like to thank all the people I worked with, that despite not being directly involved in the work presented in this thesis contributed immensely to my growth as researcher, by trusting my work and giving me autonomy and liberty to pursue my ideas, namely Judy Cho, Inga Peter and Paula Borralho.

I need to specially thank and acknowledge my two mentors, Professor Jean-Frédéric Colombel and Professor Marília Cravo. Everything I may say to thank them will be an under-statement. Having worked with so many world-wide reputed clinicians and investigators in the field of IBD during my time at Sinai, I can say with confidence that Professor Jean-Fréderic Colombel is the most passionate researcher I have ever met. His motivation and joy conducting research is contagious and everyone working with and around him feels extremely motivated to accomplish and pursue the common projects and the goals he sets for us. He has always worked in a constructive way, teaching me and guiding me through my mistakes and acknowledging my achievements. I am still everyday amazed with the fact that he appreciates my work and forever thankful for all the opportunities he gave me, for involving me in his projects and for fostering my development and career as a researcher. I have the deepest respect, admiration and friendship for him and I will never forget the years I spent at Mount Sinai working with him; I just can hope that all the projects we are still collaborating will keep on growing and that I am up to the task of deserving working with

him. Finally, I need to thank Professor Marília Cravo. If it wasn´t for her I would not be here today writing this doctoral thesis. I need to thank her for always motivating, defending and helping me in every aspect to pursue my goals, and for sharing the interest and passion for research. If it wasn´t for her, I would have probably followed the easiest path and just dedicated myself to clinical work. Her example, being a clinician and performing research, despite all day-by-day obstacles, was inspirational, and she helped me to understand what my goals were, and always helped me to accomplish them. I consider her a friend and a driving force and I admire her immensely.

Last but not the least, I want to thank my friends, parents, sister and family for always supporting me and helping me throughout these years. Being away and facing the uncertainty and unknown was not always easy and they always were there for me. Specially, I want to thank my husband, Gonçalo Leonardo, that always, but always, supported me and always joined, teamed up with me and kept me going. Nothing of this would have been possible without his love, friendship and support. This thesis is dedicated to him and to our beautiful son, João.

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ABSTRACT / SUMÁRIO

ABSTRACT

Inflammatory bowel diseases represent a group of chronic conditions characterized by relapsing inflammation within the gastrointestinal tract. Crohn's disease and Ulcerative colitis are the 2 major forms of idiopathic inflammatory bowel disease.

IBD is an extremely heterogeneous disease in what regards disease presentation, course and prognosis. The coexistence of other diseases may influence IBD´s clinical course. For example, patients with concomitant IBD and primary sclerosing cholangitis (PSC) have a distinctive phenotype, with milder colonic disease activity, more right-sided inflammation, more extensive disease and higher incidence of colorectal neoplasia. Furthermore, the co-existence of IBD bears a poor prognosis in PSC, increasing the risk of hepatobiliary malignancy, need for liver transplant or death. The reason for this distinctive phenotype is unknown, but a deeper understanding of the mechanisms underlying it could provide further insights into disease pathogenesis.

PSC is an obstructive cholestatic disease associated with changes in the bile acid pool. Therefore, bile acids (BA) and BA receptors could play a hypothetical role. BA homeostasis is tightly regulated by the activation of BA receptors expressed in the intestine. The interaction between BA and their intestinal receptors has been shown to play a key role not only in enterohepatic circulation, but also in the regulation of inflammatory liver and intestinal responses, intestinal barrier function and antibacterial defense. Furthermore, the reciprocal interaction between BA and gut flora in the intestine may be of relevance in the context of IBD, where an imbalance between the protective and harmful bacteria (dysbiosis) has been demonstrated. Altogether, these data suggest that in PSC-IBD patients, the gut-liver axis could be involved and contribute to disease phenotype. The goal of this project is to provide new insights into this special phenotype, focusing on the interactions between BA, BA receptors, and microbiome, and studying specific aspects of the natural history of patients with PSC-IBD.

We started by studying the expression of the main bile acid receptor (FXR) in PSC-IBD versus IBD alone, and its relationship with inflammation, dysplasia and location in the colon. We observed that FXR expression is inversely correlated with neoplastic progression and

severity of inflammation in UC. Furthermore, patients with PSC-UC had diminished FXR expression in the proximal colon compared to UC patients. This finding could contribute to the higher risk of proximal neoplasia in PSC patients.

Recognizing the involvement of FXR in the control of inflammation and bacterial responses in the gut, and the important role of the gut microbiome in the pathogenesis of IBD, we next explored the differences in the mucosa-associated and stool-associated microbiota in PSC-IBD versus IBD alone. Our first work in this area compared the mucosa-associated microbiome between PSC-IBD and IBD alone patients, in different locations in the colon. Biopsies taken during surveillance colonoscopy from the terminal ileum, right and left colon from patients with PSC-IBD, IBD alone and healthy volunteers were collected and sequenced using 16s rRNA for the study of the microbiome. We observed that the overall microbiome profile was similar across multiple locations in the gut from the same individual regardless of disease status, and therefore the phenotypic differences observed between PSC-IBD and IBD alone in the right versus the left colon are not explained by variations in the gut microbiome alone. We also showed that the mucosa associated-microbiome of PSC patients was characterized by an enrichment of bacteria known to be involved in bile acid handling, and gut homeostasis, therefore suggesting indeed a role of gut flora in PSC-IBD's special phenotype.

Motivated by these findings we then sought to explore the correlations between stool microbiome and bile acid profile. For this study, we recruited Portuguese patients with PSC-IBD and IBD alone patients. All patients had extensive colitis. From each patient demographic and clinical information as well as disease clinical and endoscopic activity scores were recorded. Each patient conducted a food-frequency questionnaire and nutritional analysis as well. In this study patients collected a paired fasting stool and serum sample for bile acid analysis. The stool sample was also studied and analyzed with 16S sequencing for the characterization of the gut microbiota. In this study, we observed that patients with PSC-IBD had distinct microbiota and microbiota-stool BA correlations as compared to IBD alone. Interestingly, one of the taxa enriched in PSC-IBD in this study was the *Fusobacterium* genus, a taxon known to be involved in colorectal cancer.

Finally, we wanted to study two very clinical and important questions in the management of patients with PSC-IBD: the rates of colorectal neoplasia in PSC-IBD in the current ear of improved endoscopic surveillance, and the fate of low-grade dysplasia. These are very important questions from the clinical standpoint since there is an increasing tendency to

manage low-grade dysplasia in IBD in a conservative way with increased surveillance, since the progression to high-grade dysplasia and colorectal cancer seems to be infrequent. However, whether this premise was true for PSC-IBD as well remained to be defined. Therefore, we conducted a multicentre study on almost 300 patients with PSC-IBD and 1600 patients with IBD alone and considered their longitudinal endoscopic and pathological data. We confirmed that PSC remains a strong independent risk factor for aCRN in IBD. Furthermore, we observed that there is a faster progression to advanced colorectal neoplasia, highlight the peculiarities of PSC-IBD´s special phenotype. Therefore, our findings add further credence to current recommendations for careful annual colonoscopic surveillance in this high-risk population and consideration of colectomy once LGD is detected.

It is evident that the cross talk between the liver and the colon in PSC-IBD patients, is worth exploring as it can provide important pieces of information that could lead to the development of new strategies in the management of this disease.

Keywords: Inflammatory bowel disease, Primary Sclerosing Cholangitis, Bile Acids, Farnesoid X Receptor, Gut Microbiota

SUMÁRIO

As doenças inflamatórias intestinais (DII) representam um grupo de doenças crónicas caracterizadas por inflamação recidivante do trato gastro-intestinal. A doença de Crohn e a Colite Ulcerosa são as duas principais formas de doença inflamatória intestinal idiopática.

A DII é uma doença extremamente heterogénea em termos de apresentação da doença, curso e prognóstico. Além disso, é uma doença multissistémica, podendo fazer-se acompanhar de várias manifestações extra-intestinais (MEI). A coexistência de algumas destas outras doenças pode influenciar o curso clínico da DII. Por exemplo, doentes com DII e colangite esclerosante primária (CEP-DII) concomitante, têm um fenótipo e um comportamento clínico distintos. Estes doentes apresentam frequentemente doença cólica mais extensa (pancolites), contudo com atividade endoscópica e histológica mais ligeira e daí com curso clínico da sua doença inflamatória mais favorável. Curiosamente, e ao contrário do que se verifica na colite ulcerosa, em que a atividade inflamatória é mais marcada no cólon esquerdo, vários estudos demonstraram que na CEP-DII a atividade inflamatória é mais marcada no cólon direito. Além disso, estes doentes apresentam um risco de neoplasia coloretal significativamente aumentado, que por motivos não conhecidos, e também em contraste com a colite ulcerosa (onde a neoplasia coloretal é mais frequente no cólon esquerdo), é mais frequente no cólon direito. A razão para este fenótipo distinto permanece desconhecida, mas uma compreensão mais profunda dos mecanismos subjacentes poderia fornecer mais informações sobre a patogénese da doença.

A CEP é uma doença colestática obstrutiva, associada com alterações no pool de sais bilares. Assim, os ácidos biliares (AB) e os recetores dos AB podem ter um papel hipotético. A homeostasia dos AB é fortemente regulada pela ativação de recetores expressos no intestino. A interação entre AB e os seus recetores intestinais mostrou desempenhar um papel fundamental não apenas na circulação entero-hepática, mas também na regulação das respostas inflamatórias no fígado e intestino, função de barreira intestinal e defesa antibacteriana. Além disso, a interação recíproca entre os AB e a flora intestinal podem ser particularmente relevante no contexto da DII, onde um desequilíbrio entre bactérias protetoras e prejudiciais (disbiose) foi demonstrado. Globalmente, estes dados sugerem que em doentes com CEP-DII o eixo fígado-intestino pode estar envolvido e contribuir para o

fenótipo da doença. O objetivo deste projeto é fornecer novos conhecimentos sobre este fenótipo especial, com um foco nas interações entre AB, recetores dos AB e microbioma, assim como estudar aspetos específicos da história natural da CEP-DII.

Começámos por estudar a expressão do recetor principal dos ácidos biliares, o recetor Farnesóide X, em doentes com CEP-DII e DII, em relação com o grau de inflamação no cólon, a localização no cólon e os vários graus de displasia e cancro colorectal. Para o efeito recuperámos amostras em parafina armazenadas no *Hospital Mount Sinai* em Nova Iorque e realizámos imuno-histoquímica para este recetor. Verificámos que existe um gradiente proximal-distal da expressão do recetor ao longo do cólon, uma vez que significativamente mais amostras do cólon direito apresentavam marcação forte em relação com amostras do cólon esquerdo. Além disso, verificámos que quer em doentes com DII e doentes com CEP-DII havia uma correlação negativa da expressão do recetor com a gravidade da atividade inflamatória no colon. De forma interessante, verificámos que em doentes com CEP-DII, o recetor se encontrava globalmente diminuído no cólon direito, mesmo na ausência de inflamação ativa. Finalmente, em amostras com displasia observámos uma marcação diminuída do recetor inversamente proporcional ao grau da lesão. Tendo em conta o papel do FXR na carcinogénese coloretal, os nossos dados sugerem que a sub-expressão deste marcador poderia estar envolvida no processo de carcinogénese acelerada na CEP-DII. Além disso, reconhecendo o envolvimento do FXR no controle da inflamação e regulação das respostas bacterianas no intestino, assim como o papel importante do microbioma intestinal na etiopatogenia da DII, decidimos explorar as diferenças do microbioma entre doentes com CEP-DII e DII. O primeiro trabalho realizado nesta área foi realizado em doentes que se apresentavam para realização de colonoscopia de vigilância (CEP-DII e DII) e voluntários saudáveis. Atendendo às várias diferenças entre cólon direito e cólon esquerdo em doentes com CEP-DII e DII, estávamos também interessados em explorar se diferenças na composição do microbioma associado à mucosa poderiam estar envolvidos neste aspeto do fenótipo. Assim, foram colhidas biopsias adicionais para isolamento de DNA bacteriano e sequenciação do gene 16rRNA. Observámos que não havia diferenças significativas entre a composição microbiana entre os diferentes locais do cólon, pelo que as diferenças fenotípicas observadas entre CEP-DII e a DII entre o cólon direito e esquerdo não são explicadas pelas variações do microbioma intestinal. Contudo verificámos que o microbioma da mucosa de doentes com CEP-DII se caracterizava por enriquecimento de

grupos bacterianos que se sabe estarem envolvidos no metabolismo dos sais biliares, sugerindo, portanto, um eventual papel da flora intestinal, no fenótipo especial da CEP-DII. Motivado por esses achados, procurámos explorar as correlações entre o microbioma fecal e o perfil de AB fecais. Para esse efeito recrutámos doentes portugueses com CEP-DII e DII apenas. Todos os pacientes tinham colite extensa. Recolhemos informações demográficas, clínicas, assim como os *scores* de atividade clínica e endoscópica de todos os doentes. Além disso, cada doente realizou também um questionário de frequência alimentar e análise nutricional. Todos os doentes colheram uma amostra de soro e fezes para análise de ácidos biliares. A amostra de fezes também foi estudada e analisada com o recurso a sequenciação 16S para a caracterização do microbioma intestinal. Neste estudo, verificámos que embora não existissem diferenças estaticamente significativas na proporção de AB fecais individuais, o *pool* de AB era globalmente distinto entre a CEP-DII e a DII. Além disso, comprovámos que os doentes com CEP-DII apresentavam interações distintas entre o microbioma e os sais biliares fecais em comparação com os doentes com DII apenas. Curiosamente, um dos grupos taxonómicos enriquecidos na CEP-DII neste estudo foi o género *Fusobacterium*, previamente descrito como estando envolvido no cancro coloretal.
Finalmente, pretendemos estudar duas questões clínicas de especial relevância no manejo de pacientes com CEP-DII: as taxas de neoplasia coloretal numa era de melhor vigilância endoscópica e o grau de progressão da displasia coloretal de baixo grau. Estas são questões muito importantes do ponto de vista clínico, uma vez que na DII há uma tendência crescente para o manejo de displasia de baixo grau de forma conservadora, uma vez que a progressão para displasia de alto grau e cancro coloretal parece ser pouco frequente. No entanto, permanece por esclarecer se esta premissa também se pode aplicar em doentes com CEP-DII, que têm um risco basal muito elevado. Assim, realizámos um estudo multicêntrico com quase 300 doentes com CEP-DII e 1600 doentes com DII, e estudámos os seus dados endoscópicos e histopatológicos longitudinais. Confirmámos que, apesar de todos os avanços no tratamento da DII e nas técnicas de vigilância endoscópica, a CEP continua a ser um fator de risco independente para o desenvolvimento de displasia de alto grau e cancro coloretal. Além disso, observámos que, quando existe o diagnóstico displasia de baixo grau, existe uma rápida progressão para a neoplasia coloretal avançada, destacando mais uma vez as peculiaridades do fenótipo especial da CEP-DII. Assim, os nossos achados reforçam as recomendações atuais de vigilância endoscópica anual cuidadosa nesta população de alto risco e consideração de colectomia, uma vez diagnosticada displasia de baixo grau.

Deste conjunto de estudos é pois evidente que o *cross-talk* entre o fígado e o cólon em doentes com CEP-DII, pode fornecer informações importantes que poderiam levar ao desenvolvimento de novas estratégias na abordagem desta doença.

Palavras-chave: Doença Inflamatória Intestinal, Colangite Esclerosante Primária, Sais biliares, Recetor Farnesóide X, Microbiota Intestinal

ABREVIATIONS

Abbreviation	Description
5-ASA	5-Amynosalicilates
AIH	Auto-Immune Hepatitis
ALP	Alkaline Phosphatase
AOM	Azoxymethan
APC	Adenomatous Polyposis Coli
ASBT	Apical Sodium Bile Acid Transporter
ATG16L1	Autophagy-Related Protein 16-1 gene
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CA	Cholic Acid
CCA	Cholangiocarcinoma
CD	Crohn´s Disease
CDCA	Chenodeoxycholic Acid
CRC	Colorectal Cancer
CRN	Colorectal Neoplasia
CRP	C-Reactive Protein
CYP7A1	Cholesterol 7-A-Monooxygenase
DAB	Diaminobenzidine
DCA	Deoxycholic Acid
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle's Medium
DSS	Dextrane Sodium Sulfate Models
EIM	Extra-Intestinal Manifestations

ERCP	Endoscopic Retrograde Cholangiopancreatography
FBS	Fetal Bovine Serum
FDR	False-Discovery Rates
FGF19	Fibroblast Growth Factor-19
GGT	Gammaglutamyl Transpeptidase
GWAS	Genome Wide Association Studies
HAI	Histological Activity Index
HER	Electronic Health Record
HGD	High-Grade Dysplasia
HLA	Human Leukocyte Antigen
HPLC	High-Performance Liquid Chromatography
HR	Hazard ratios
IBABP	Ileal BA-Binding Protein
IBD	Inflammatory Bowel Disease
IBDU	Inflammatory Bowel Disease Unclassified
ICD	International Classification Of Disease
IFN-γ	Interferon gamma
Il	Interleukin
IM	Immunomodulator
IQR	Interquartile range
IRGM	Immunity-related GTPase family M protein nege
JAK2	Janus Kinase
LC	Left colon
LC-MS/MS	Liquid Chromatography Coupled with Tandem Mass Spectrometry

LCA	Lithocolic acid
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis effect size
LGD	Low-grade dysplasia
LRKK2	Leucine-rich repeat kinase 2 hene
MAPK	Mitogen-activated protein kinase
MRCP	Magnetic resonance cholangiopancreatography
mRNA	Messenger RNA
MUC2	Mucin 2 gene
NFKB	Nuclear factor KB-dependent
NGS	Next-generation sequencing
NK	Natural killer
nMDS	Non-metric multiple dimensional scaling
NOD	nucleotide-binding oligomerization domain
NPR	National Patient Registry
OLT	Orthotopic liver transplantation
OR	Odds ratio
OST	Basolateral organic solute transporter
OTU	Operational taxonomic unit
PANDAseq	PAired-eND Assembler for DNA sequences
PBS	Phosphate Buffered Saline
PCA	Principal component analysis
PCoA	Principle coordinates analysis
PCR	Polymerase chain reaction

PD	Phylogenetic Diversity
PerMANOVA	Permutational Multivariate Analysis of Variance
PRDX5	Peroxiredoxin-5 gene
PSC	Primary Sclerosing Cholangitis
QIIME	Quantitative insights into microbial ecology
RC	Right colon
sdPSC	Small-duct PSC
SES-CD	Simple endoscopic score for CD
SHARE	Sinai-Helmsley Alliance for Research Excellence Network
STAT3	Signal transducers and activators of transcription 3 pathway
T_H	T helper
TI	Terminal ileum
TLR	Toll-like receptor gene
TMAs	Tissue microarrays
TNBS	Trinitrobenzensulfonic acid
TNF	Tumor necrosis factor
T_{REG}	T regulatory cells
UC	Ulcerative colitis
UDCA	Ursodeoxycholic acid
UPGMA	Unweighted pair group method with arithmetic mean

CHAPTER 1

INFLAMMATORY BOWEL DISEASE ASSOCIATED WITH PRIMARY SCLEROSING CHOLANGITIS: A SPECIAL PHENOTYPE

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) represents a group of chronic conditions characterized by relapsing-remitting inflammation within the gastrointestinal tract. There are two major types of IBD: Crohn´s disease and Ulcerative colitis. All ages can be affected, but IBD typically affect young patients. Being a chronic disease with a young age of onset, IBD has a major impact on the personal and professional life of patients. It can lead to progressive bowel damage and disability, with a significant number of patients needing surgery over time[1, 2]. Besides the intestinal manifestations, other organs and several types of extra-intestinal manifestations can also affect patients and have an impact in quality of life and prognosis.

Clinical symptoms and diagnosis

Clinical presentation varies depending on the segment affected and type of involvement. In Crohn's disease, any segment of the GI tract, from the mouth to the anus, can be affected but most frequently disease is located in the terminal ileum and proximal colon. The inflammation in CD is typically asymmetric, segmental and transmural[1]. Three patterns of involvement can be seen: inflammatory, stricturing and fistulizing. The typical symptoms in CD are abdominal pain, chronic diarrhea and other features suggestive of IBD (eye, skin, joints complaints, perianal manifestations and family history of IBD). Weight loss, anorexia, fatigue and low-grade fever are frequently present, independent of disease location. In some patients, subclinical inflammation over the years, results in fibrotic strictures, and postprandial abdominal pain, distension and vomiting may be the presenting complaints. Due to the transmural nature of inflammation patients can present with abscesses, inflammatory masses, or fistulae to adjacent organs or skin (**Figure 1**). Perianal disease occurs in almost 1/3 of patients and may be the chief complaint[3].

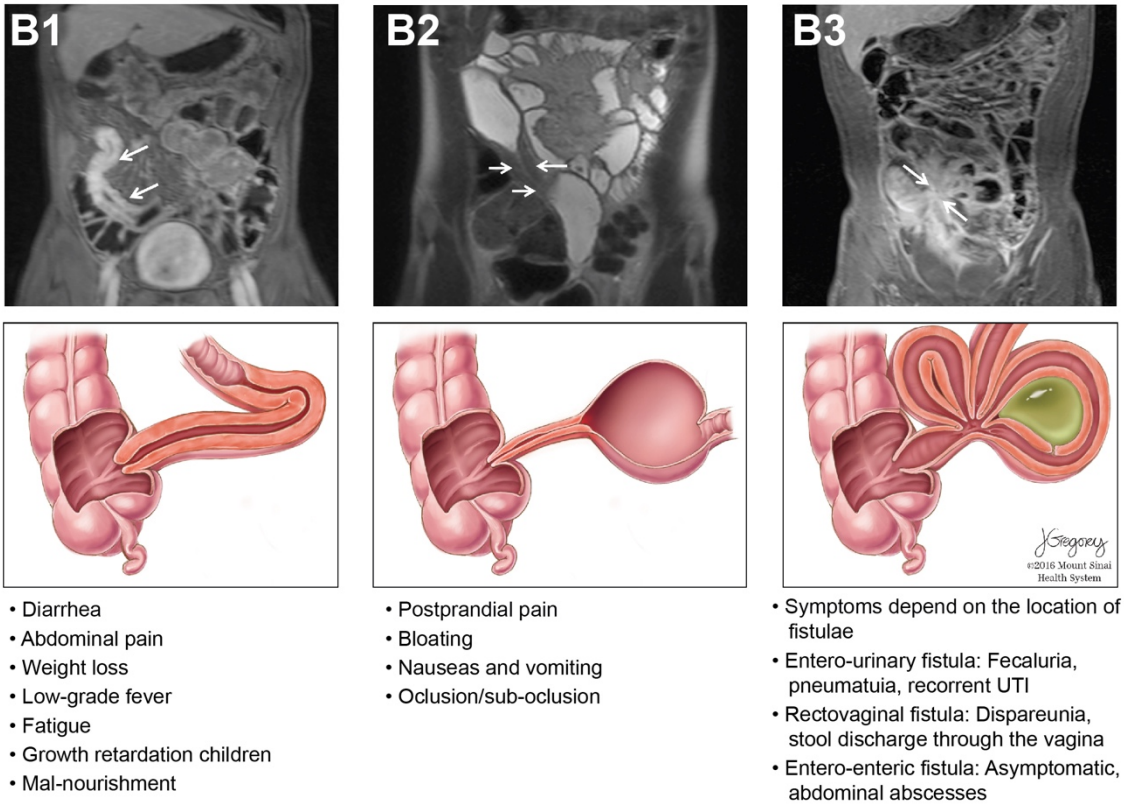


Figure 1 – Crohn´s disease classification based on disease behaviour. The figure depicts the three types of behaviour of Crohn´s disease as per the Montreal classification represented in magnetic resonance enterography (MRE) and illustrated, with typical symptoms. Upper left MRE (B1) shows mural thickening and enhancement in the distal ileum in a patient with active CD (T1 weighted imaging with fat saturation after injection of gadolinium chelates). The middle MRE panel (B2) shows a narrowed luminal segment with thickened wall and upstream dilation suggesting the presence of a stricture (T2 weighted imaging). The right MRE panel (B3) shows multiple converging enhancing loops of small bowel suggestive of entero-enteric fistulae (arrows) (T1 weighted imaging with fat saturation after injection of gadolinium chelates); in the lower illustration, a deep and transmural fissure/ulcer leads to the formation of an abscess. *Reproduced with permission from Torres J et al. (2017) Crohn´s disease. The Lancet.* doi: 10.1016/S0140-6736(16)31711-1[1].

In UC, inflammation is usually restricted to the colon only, involving only the mucosa and sometimes the sub-mucosal layers; usually the inflammatory process starts in the anal canal and extends proximally. Patients may have disease limited to the rectum (proctitis), up to the splenic flexure of the colon (left-sided colitis) or beyond the splenic flexure (extensive colitis) (**Figure 2**)[1]. This classification has prognostic implications, since more extensive

forms of disease (left-sided colitis, pancolitis) are usually associated with higher need for steroids, hospitalization and surgery[4, 5].

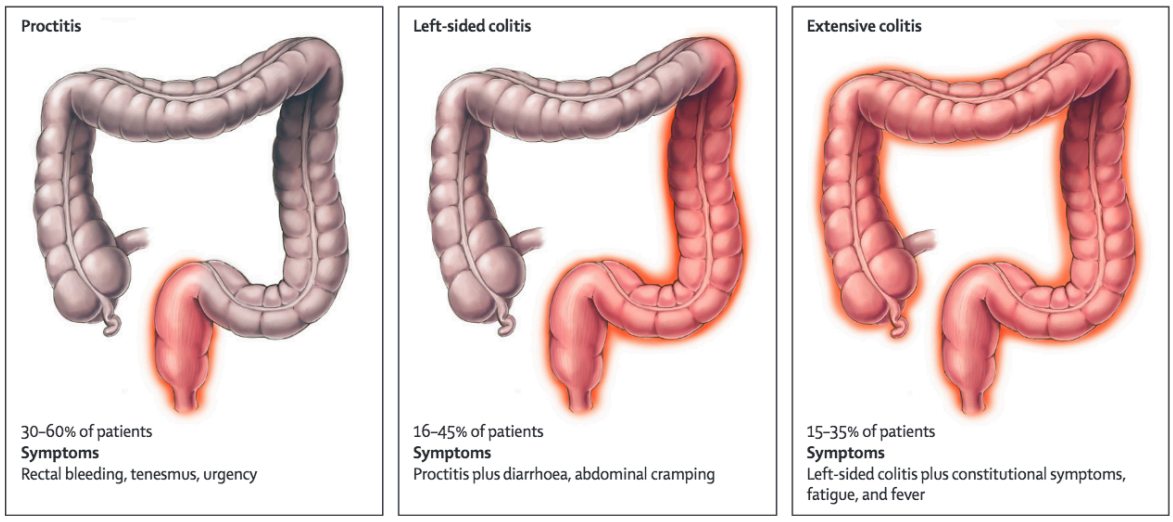


Figure 2 – Ulcerative colitis classification based on disease extent. The figure depicts the 3 types of mucosal involvement usually seen in ulcerative colitis, as per the Montreal classification. This classification has prognostic and therapeutic implications, since proctitis is usually associated with a milder disease course, lower therapeutic requirements, less need for hospitalizations and surgery[4, 5]. *Reproduced with permission from Ungaro R et al. (2017) Ulcerative colitis. The Lancet.* doi: 10.1016/S0140-6736(16)32126-2[6].

Occasionally mild inflammation can be seen in the terminal ileum, a process known as backwash ileitis. Typical symptoms in UC include rectal bleeding, bloody diarrhea, fecal urgency and incontinence, mucus discharge, and abdominal pain. Clinical presentation might vary based on disease extent. Patients with proctitis might predominantly have urgency and tenesmus (sensation of incomplete evacuation), while in pancolitis, bloody diarrhea and abdominal pain might be more prominent.

The diagnosis of IBD relies on a combination of clinical, laboratorial, endoscopic, histological, and radiological findings after exclusion of alternative diagnosis[1]. Endoscopy with biopsies is required to establish the diagnosis. Typical endoscopic findings in CD are ulcerations interspersed with areas of normal mucosa, cobblestoning, and rectal sparing. Focal, discontinuous and segmental chronic inflammation is the key pathological finding. Non-caseating granulomas can sometimes be found[1, 7] . In UC, typical endoscopic findings are erythema, loss of normal vascular pattern, granularity, erosions, friability, bleeding, and

ulcerations. The typical histologic findings are distortion of crypt architecture, increased lymphocytes and plasma cells in the lamina propria, basal plasmocytosis, mucin depletion, and Paneth cell metaplasia[67].

IBD is a multisystemic disease, and multiple other organs can be affected, including the bones, joints, mouth, skin, eyes, hepatobiliary system, lungs and kidneys[8-10]. The overall prevalence of these extra-intestinal manifestations (EIM) ranges from 20-50%, and they may present prior, in conjunction or following the diagnosis of the bowel disease[8]. Some EIM run in parallel with the bowel disease activity while other have an independent course. One of such EIMs is **Primary Sclerosing Cholangitis (PSC)**, described for the first time in 1965[11]. Patients with IBD and concomitant PSC have a distinct phenotype and disease course (see below).

Epidemiology

IBD is a chronic disease that can affect all ages. There is no sex-specific distribution in adult IBD, and the onset of the disease usually occurs in the 2nd- 4th decade of life, with the highest incidence reported among 20 to 29 year-old individuals; a second and smaller peak has been described inconsistently between 50-60 years[12]. There has been a steady increase in IBD frequency in most regions of the world[12]. Usually there is a rise in the incidence of UC, followed by a rise in the number of new cases of CD[12]. The incidence and prevalence of IBD is highest in westernized nations, and in urban compared to rural areas. The highest reported incidence rates come from Canada (20.2/10^5), Northern Europe (10.6/10^5), New Zealand (16.5/10^5) and Australia (29.3/10^5)[12]. Prevalence rates are highest in Europe (322/10^5)[9], Canada (319/10^5)[4] and United States (214/10^5)[1, 13-16]. Portugal is considered to be an intermediate incidence and prevalence region, with a growing number of new cases[16].

Etiology and pathophysiology

Genetics and family history

Several lines of evidence link genetics to the risk of IBD. First, around 12% of patients have a family history of IBD, with the risk being higher if first-degree relatives or multiple family members are affected[17, 18]. Second, concordance rates in monozygotic twins range from 20–50%, with higher concordance rates for CD than UC[19]. Third, certain ethnic groups are specifically affected- the risk of IBD is 3-4 times higher in the Ashkenazi Jewish[19], while

African-American and Asian ancestry is associated with the lowest risk[19]. Finally, Genome-wide association studies (GWAS) have identified more than 200 risk alleles associated with IBD, of which 37 are specific for CD and 32 for UC[20]. Interestingly, 70% of the loci are shared with other diseases, such as type 1 diabetes, ankylosing spondylitis and psoriasis[20]. Furthermore, there is a striking overlap between loci for IBD and primary immunodeficiencies and mycobacterial infection. Among the identified genes, notable examples include mutations in genes associated with bacterial sensing and innate immunity (NOD2, ATG16L1, LRKK2, IRGM, Il23R, HLA, STAT3, JAK2 and Th17-pathway) and altered mucous layer (MUC2)[21]. However, only 13.1% of disease heritability is explained by genetic variation, highlighting the importance of epigenetic and other non-genetic environmental factors[1].

Environmental factors

In the past years, areas of the world where IBD was previously considered to be very rare, have witnessed a sharp increase in their incidence rates, almost in parallel with the fast industrialization rates ongoing in these areas[1, 22]. Changes in lifestyle, diet, better sanitation and Westernization" of lifestyles has been proposed as a potential explanation to the rise of allergic and immune-mediated disorders, including IBD over the past few decades ('hygiene hypothesis'). However, breastfeeding, living in rural environments, contacting with animals in childhood, etc, have been only inconsistently identified as being "protective" for IBD[1, 23, 24]. Cigarette smoking is the best studied environmental factor; it is associated with twofold increase in risk for developing CD (OR, 1.76; 95% CI 1.40-2.22), while it is protective from developing UC[1, 6, 25-27]. Appendectomy performed before the age of 20 years confers protection against ulcerative colitis[28]. Antibiotic exposure in early childhood, and during pregnancy has been shown to increase the risk of IBD[29, 30], again highlighting a putative role for the microbiota in disease pathogenesis. Other medications potentially associated with higher risk of developing IBD include oral contraceptives, aspirin and nonsteroidal anti-inflammatory drugs, while statins have been linked with a decrease in the incidence of IBD, especially in the elderly[31, 32]. A reduction in dietary fiber and an increase in saturated fat intake have also been associated with increased risk[33-35]. Micronutrients such as zinc or iron, and Vitamin D have been proposed to potentially play a role. Causative association remains

to be proven for many environmental factors, as only association studies with major methodological limitations have been conducted.

Microbiota

The role and involvement of the gut microbiota in the pathogenesis of IBD has long been supported by many clinical observations, such as disease location in areas of highest bacterial population, the positive role of antibiotics for inducing remission and for preventing post-operative recurrence, and remission of disease upon diversion and recurrence upon re-anastomosis[36]. The advent of next-generation sequencing, allowed the characterization of an abnormal composition of commensals called "dysbiosis". Generally, IBD is characterized by a decrease in species diversity, namely in *Bacteroides* and *Firmicutes*, and specifically in bacteria from the *Clostridium cluster XIVa and IV,* and an increase in Gamma*proteobacteria*[37] and *Actinobacteria*[38, 39]. *Faecalibacterium prausnitzii*, a butyrate-producing commensal with anti-inflammatory properties, has been shown to be reduced in mucosal samples from patients with CD and UC[38, 39]. Approximately 1/3 of CD patients display in ileal biopsies increased numbers of mucosa-associated *Escherichia coli*, designated adherent-invasive *E. coli* (AIEC)[30,31] . These *E.coli* strains are able to cross the mucus barrier, adhere and invade intestinal epithelial cells (IEC), and survive and replicate within macrophages, provoking the secretion of high amounts of TNF-α[30,31]. The potential causative role of the gut microbiota in disease pathogenesis, had led many investigators to seek a microbial-derived therapy. So far the experience with antibiotics and probiotics has been disappointing[40], but the recently published results showing a promising role for fecal transplant in the setting of ulcerative colitis has again led to very active investigation in this area[41].

Intestinal immune system in IBD

IBD is a complex disease with a complex ethiopathogenesis. It is commonly accepted that IBD results from the interplay between environmental factors, genetic susceptibility, and intestinal microflora resulting in an abnormal mucosal immune response and compromised epithelial barrier function. Multiple and overlapping pathways of the intestinal immune system are dysregulated in IBD (**Figure 3**).

The mucous barrier and epithelial barrier defects are strongly implicated in the pathogenesis of UC and CD. The epithelium establishes a "buffer zone" about 50 μm thick between the

luminal contents and itself[42]. Defects in the barrier function of the intestinal mucosa can prime increased microbial and antigen presentation and lead to immune activation. After triggering by antigen presentation, both innate and acquired immune responses are activated with subsequent loss of tolerance to enteric commensal bacteria. This results in sustained Th1 and/or Th17 responses and production of pro-inflammatory cytokines (Il1, Il2, Il6, INF-γ, TNF-α, Il17, etc.). TNF-α is one of the most relevant mediators in intestinal inflammation. Together with Il1 and Il6 it also contributes to symptoms such as fever, anorexia and weight loss. Disruption of this buffer zone by emulsifiers, ubiquitous in western diet[43] or by mutations in MUC2 gene[44] are associated with IBD. Further, epithelial cells are armed with an evolutionarily conserved process called autophagy, in which unwanted cytoplasmic contents are targeted to the lysosome for degradation, preventing the dissemination of invasive bacterial species. Notably, defects in autophagy-related genes such ATG16L1 and IRGM have been identified as further important risk factors for the development of CD[45]. Finally, defects in intestinal tight junctions, comprising members of the claudin and occludin families, are associated with IBD[3]. Many studies performed in patients with IBD have shown that intestinal barrier function is disrupted both in active and in quiescent disease states. Indeed, several studies have shown that increased intestinal permeability to inert tracer molecules such as PEG molecules or ^{51}Cr-EDTA occurs before inflammation and can predict disease relapse in patients with CD[46]. Interestingly, increased permeability has also been documented in first-degree relatives of patients with CD, underlining possible shared genetic susceptibility to disease[17].

An array of innate immune mechanisms coordinates to preserve mucosal function and integrity. The NOD-like receptors (NLRs) are one such class of innate immune proteins that mobilize host defense to intracellular fragments of bacterial peptidoglycan, by initiating nuclear factor KB (NFKB)-dependent and mitogen-activated protein kinase (MAPK)-dependent gene transcription, producing multiple protective cytokines. NOD2 was the first gene to be associated with CD[47] and it remains a prominent genetic risk factor for CD. Dendritic cells (DCs), key antigen-presenting cells, are tolerogenic at steady state. However, under inflammatory conditions they transform into cells with inflammatory potential. In patients with IBD, intestinal DCs have enhanced expression of TLR2, TLR4, and co-stimulatory molecules, and they secrete increased pro-inflammatory cytokines compared to healthy controls. Intestinal macrophages, play essential housekeeping functions, such as the clearance of apoptotic or senescent cells and tissue remodeling at steady state[48]. Finally,

neutrophils choreograph the early response to microbial stimuli and likely modulate the adaptive responses beyond the acute state by the production of cytokines and reactive oxygen species (ROS). Adaptive immune cells have long been implicated in the pathogenesis of IBD. $CD4^+$ T helper (T_H) cells can be functionally compartmentalized into T_H1, T_H2, T_{REG}, T_H17, T_{FH} and T_{H9} cells[49]. Broadly, ulcerative colitis is a modified T-helper-2 (Th2) disease, while Crohn's disease is Th1 driven disease. Intestinal inflammatory infiltrate in CD contains both T_H1 and T_H17 cells. Such effector T cell responses to bacteria or fungi are implicated in the pathogenesis of CD[49]. In addition, impaired functional activity of intestinal T_{REG} has been reported in CD patients[50]. The colonic lamina propria cells from patients with ulcerative colitis contains Th2-T cells that produce interleukin-5 (IL5) [49]. Il4 and IL13 have also been implicated in the pathogenesis of ulcerative colitis. IL13, produced by non-classical natural killer T cells is a key mediator of epithelial cytotoxicity and barrier dysfunction in ulcerative colitis[49].

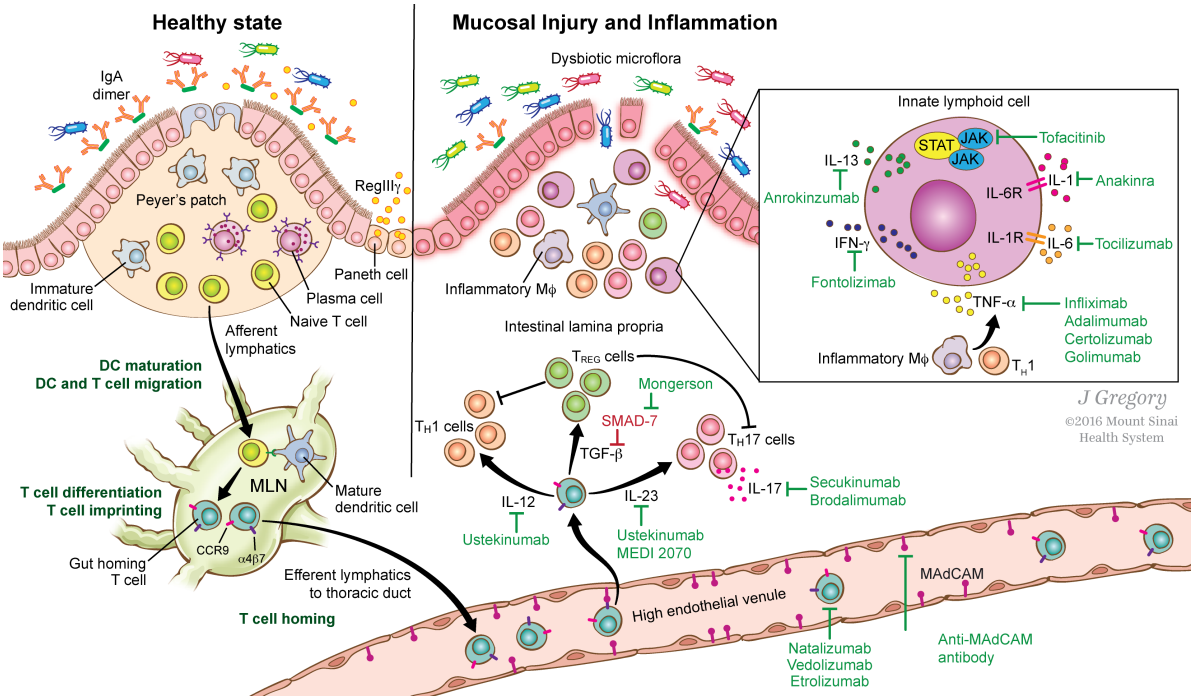


Figure 3 - Overview of the intestinal immune system in health and IBD. During the healthy state (left side of the figure), the intestinal epithelium and IgA dimers work in concert to regulate and separate the luminal microflora from the mucosal immune system. During the healthy state, barrier function is maintained by the mucus layer and epithelial cells bound across tight junctions The intestinal epithelium also contains specialized cells such as Paneth cells (that produce antimicrobial peptides) and M cells (that sample luminal antigens). M cells are in close contact with antigen

presenting cells such as dendritic cells (DC). Contact with the antigen leads to DC maturation and antigen presentation to T and B cells. DCs default to inducing a "tolerizing" phenotype in the mucosa unless danger signals such as bacterial LPS induce the switch to an inflammatory/immunizing DC phenotype. Intestinal DCs also imprint T and B lymphocytes to express gut homing molecules α4β7 and CCR9. Lymphocytes thus imprinted within the GI tract enter the systemic circulation and upon reaching intestinal high endothelial venules, the gut-imprinted, α4β7-expressing lymphocytes engage locally expressed MAdCAM and egress the circulation to enter the intestinal lamina propria. The intestinal lamina propria has multiple families of T cells. These include TH1, TH17 and TREG subtype. At steady state TREG regulate the activity of TH1 and TH17 cells and prevent unchecked inflammation. During mucosal injury and inflammation such as seen in Crohn's Disease (right side of the figure), the epithelial barrier is breached as a primary or secondary event and the luminal microflora stimulates a pro-inflammatory immune response by DCs and inflammatory macrophages (Mφ). The regulatory ability of TREG is outstripped by inflammatory activity of TH1 and TH17. Additionally, innate lymphoid cells (inset), homeostatic at steady state contribute to the cytokine production, perpetuating inflammation. Mucosal injury and damage is associated with dysbiosis, which perhaps perpetuates the inflammatory cascade. Increasing understanding of the mucosal immune system has led to an expanding array of therapeutic targets. Of these, TNF-α antagonists and homing inhibitors are currently in clinical practice while the others are in early to advanced stages of clinical development. DC= dendritic cell, MAdCAM=Mucosal addressin Cell Associated Molecule, IL=interleukin, TH= T helper cell, TREG= Regulatory T cell, IFN= interferon, Mφ= macrophage, TGF= transforming growth factor. Illustration by Jill Gregory. *Reproduced with permission from Torres J et al. (2017) Crohn´s disease. The Lancet.* doi: 10.1016/S0140-6736(16)31711-1[1].

The management of IBD

The enormous advances in the knowledge about disease pathogenesis, resulted in the development of new targeted therapies directed at specific cellular processes, that led to major improvements in the care of patients. This resulted in a treatment paradigm change that evolved in from mere control of symptoms and improved quality of life towards attaining deep remission (absence of symptoms and endoscopic healing), with the goal of blocking the natural progression of disease and avoiding complications, bowel damage and disability[5, 51]. However, there is no cure for disease, and medical-free remission is not possible in most patients[52].

The choice of medication depends on disease location, behavior, and severity and response to previous therapies, and considers prognostic factors that are known to be associated with higher probability of complicated disease[41]. The treatment of IBD involves an induction and maintenance regimen. The most widely used drugs in IBD currently are corticosteroids, 5-aminosalicilates, immunosuppressants (IM) [thiopurines (azathioprine, mercaptopurine), and methotrexate], biologics [(anti-TNF (infliximab, adalimumab and golimumab), anti-adhesion molecules (vedolizumab) and anti-IL23 (ustekinumab)][1, 3, 6]. Corticosteroids are potent anti-inflammatory drugs that are used to rapidly induce clinical remission; they should never be used as maintenance therapy, since they have multiple side-effects. Patients who are dependent on steroids to maintain clinical remission have indication to start steroid-sparing agents such as immunomodulators or biologic drugs[1, 3, 6]. The 5-aminosalicylates drugs are not effective in the treatment of Crohn´s disease but are usually the first-line therapy for the treatment of mild UC (administered as suppositories, enemas, or oral formulations). Antibiotics use should be restricted to CD complicated by fistulas and/or abscesses. Biological drugs are usually reserved for patients with moderate-severe UC. There is an increasing tendency to consider early aggressive therapy (IM and/or anti-TNF) in the subgroup of patients with poor prognostic factors (perianal disease, extensive small bowel disease, young age at diagnosis, deep ulcers in endoscopy, penetrating disease) complicated and/or severe disease (top-down therapy)[1, 3, 5, 6]. Patients with refractory medical disease, who develop complications (abscesses or malignancy), and/or do not tolerate medical therapy are candidates for surgery[1, 3, 6]. The decision to advance for surgery should always be discussed in the context of a multidisciplinary team involving dedicated surgeons and gastroenterologists, and should include appropriate pre-operative imaging, patient counselling, optimization of nutritional status of the patient, and reduction in the thromboembolic risk[1, 6].

PRIMARY SCLEROSING CHOLANGITIS

PSC is a chronic and progressive cholestatic disease, characterized by inflammation and fibrosis of the intrahepatic and/or extrahepatic ducts[53], that may result in liver cirrhosis and eventually end-stage liver disease[53]. Orthotopic liver transplantation (OLT) is the only potentially curative therapy for PSC, with survival rates of 85% and 70% at 5 and 10 years,

respectively[53]. Without OLT, half of symptomatic patients die within 12-15 years. In Western countries, the reported incidence of PSC is 0.07-1.3 per 100.000/year, and the prevalence is 8.5-13.6 per 10.000[54,55]. There is no epidemiological data available in Portugal for PSC, but it is generally considered a very rare disease.

Having a diagnosis of IBD is the strongest risk factor for PSC development, since 70% of patients with PSC have underlying IBD, most frequently ulcerative colitis (UC) in over 56-72%% of cases[56, 57]. Conversely, in patients with known IBD, PSC is found much less commonly, occurring in about 2-8% of UC patients and 3% of Crohn's disease (CD) cases[58].

Although there may be a possible common pathogenesis between PSC and IBD, the two disorders can occur at different times. PSC may be diagnosed many years after proctocolectomy for colitis, and conversely IBD can appear many years after the initial diagnosis of PSC or even after OLT altogether[59]. In most reports, IBD diagnosis precedes the diagnosis of PSC[60, 61]. In patients with known IBD, the presence of persistent of unexplained cholestasis obliges one to exclude concurrent PSC through magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP). When PSC is diagnosed first, half of the cases have only abnormal laboratory tests; the typical diagnostic hallmarks of fever, itching, and jaundice are rarely seen nowadays[53]. Symptomatic patients usually present with fatigue and pruritus and can also exhibit jaundice, hepato-splenomegaly or scratching injuries. Recurrent episodes of bacterial cholangitis with fevers, chills, right upper quadrant pain and jaundice can also be a part of the clinical presentation, and usually develops in about 10–15% of patients during the course of their disease[62]. The diagnosis of PSC is based on the findings of diffuse multifocal strictures and dilations in the intrahepatic and/or extrahepatic biliary tree[53] (**Figure 4**).

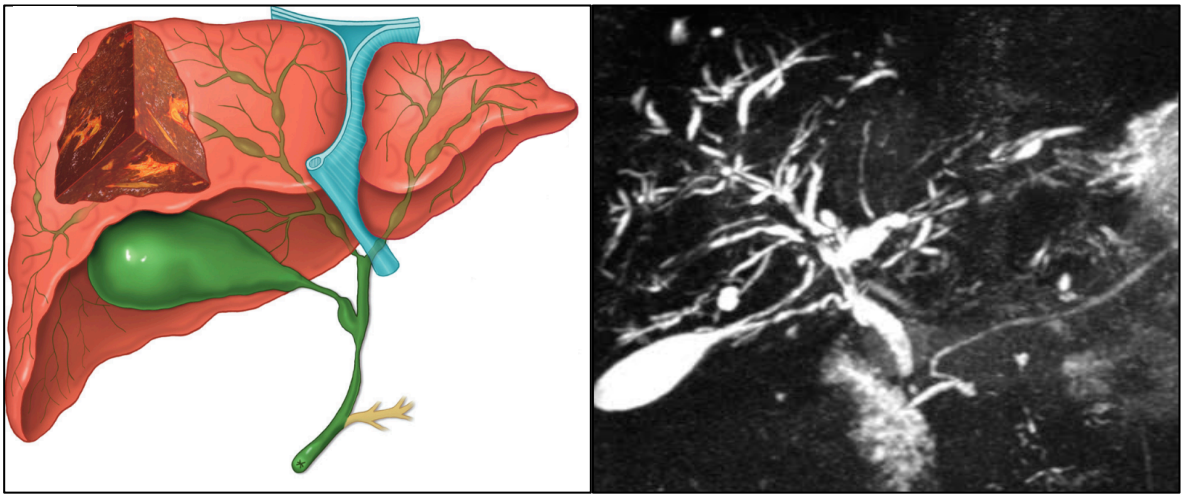


Figure 4 – Typical findings of Primary Sclerosing Cholangitis. Diagram (left) and magnetic resonance cholangiopancreatography imaging (right) showing the typical findings of primary sclerosing cholangitis. Several strictures with intervening saccular dilatations of both the intrahepatic and the extrahepatic bile are seen conferring a beading aspect to the intra-hepatic biliary tree. Diagram in the left reproduced with permission from *Hirschfield G et al. (2013) Lancet. Primary Sclerosing Cholangitis.* doi: 10.1016/S0140-6736(13)60096-3[63]. Cholangiogram-MRI image courtesy of Afonso Gonçalves, MD

Patients with a confirmed diagnosis of PSC should undergo colonoscopy with biopsies to exclude concomitant IBD or any malignancy[53], even if they report no gastrointestinal symptoms. As most of PSC-IBD patients have mild colonic disease activity and even possible normal endoscopic appearances, histological sampling is crucial to avoid underdiagnosis[58]. Although no evidence-based guidelines are available, if the index colonoscopy is negative for IBD, a repeat colonoscopy every 3-5 years should be performed to monitor for possible onset of IBD[64].

The pathogenesis of PSC-IBD – what is known

PSC is likely to have an underlying multifactorial etiology, with a predominant immune-mediated process[53, 65]. PSC and IBD are interrelated conditions that may share an underlying predisposition. Both diseases share common antibodies, such as those directed against cytoplasmic and nuclear antigens of neutrophils with a characteristic perinuclear staining pattern (p-ANCA). The p-ANCA antibodies have been found in 26–85% of PSC patients

and in up to 68% of patients with UC[53]. The available evidence points towards a complex interaction between genetic, immunologic and environmental factors (**Figure 5**).

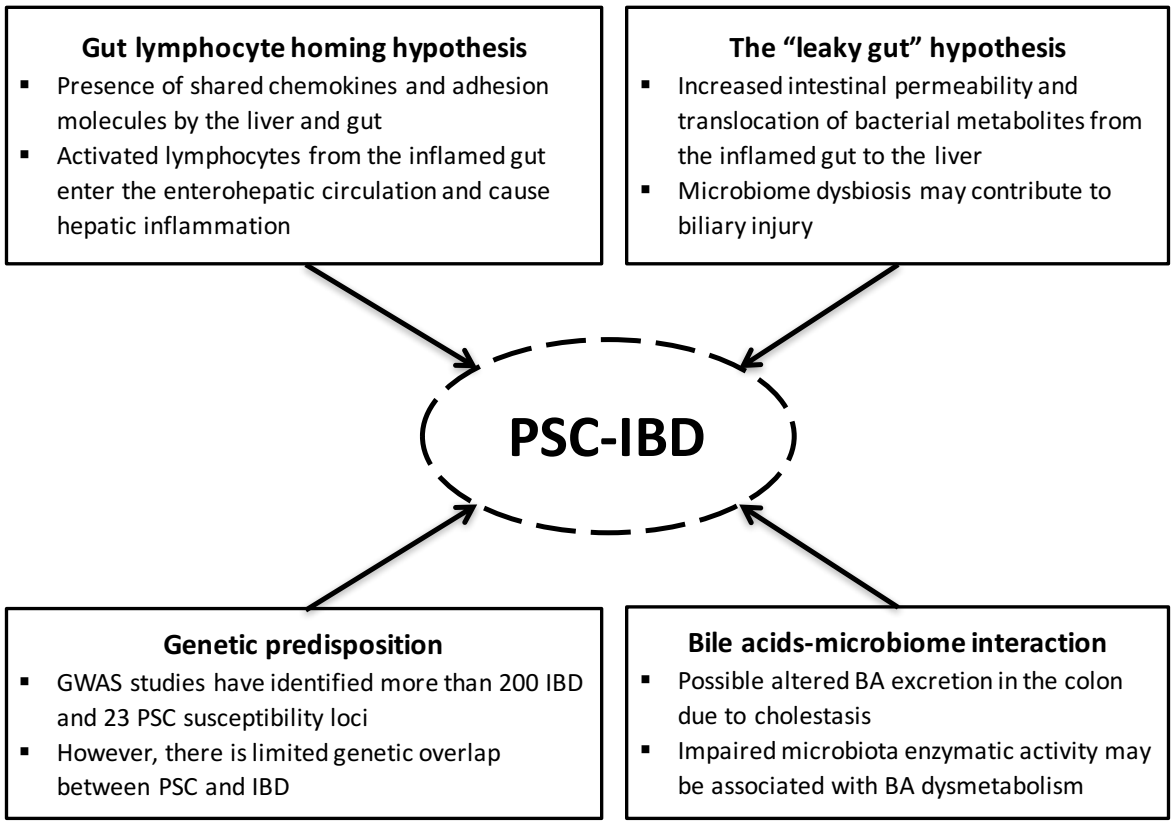


Figure 5 - Possible hypothesis linking PSC and IBD pathogenesis, including genetic predisposition, immune-mediated processes, altered gut microbiota and altered bile acids (BA) metabolism. *Reproduced with permission from Palmela C. et al (2017). Gut Liver. Inflammatory Bowel Disease and Primary Sclerosing Cholangitis: A Review of the Phenotype and Associated Specific Features. doi: 10.5009/gnl16510* [65].

Genetics

From a genetic standpoint, there is increasing evidence that PSC is distinct from UC and CD. Large-scale genome-wide association studies (GWAS) have identified close to 200 independent loci associated with IBD[20, 65, 66]. Most of these loci are shared between UC and CD[20]. Genome-wide association studies (GWAS) have allowed for the identification of 23 genetic risk loci associated with PSC.[67] Most of these genetic risk loci play an important role in the immune system, such as the HLA complex, *IL2,* or *PRDX5*, suggesting that PSC may be an immune-mediated disorder. Furthermore, there is some overlap between some genetic risk loci of PSC and IBD. However, the known genetic defects only explain less than 10%

of PSC disease predisposition[68], pinpointing the possible importance of the environment in the pathogenesis of the disease. Genetic predisposition to autoimmune bile duct injury triggered by toxic or infectious agents that may gain access through the diseased colon is potentially a major mechanism leading to PSC in IBD patients[65].

Gut microbiome

Several lines of evidence support the involvement of gut microflora in PSC´s pathogenesis. Bacteria and fungi are more frequently found in the bile ducts of patients with PSC, as compared to patients with other cholestatic liver diseases[69, 70]. Several reports and case series of pediatric patients with PSC treated with vancomycin with positive results further suggest a role for the gut microbiome, and have paved the way for the trial of antibiotics in PSC[71]. Indeed, in a pilot small randomized controlled trial where patients were allocated to vancomycin high or low doses, or metronidazole, high or low doses, it was shown that patients receiving vancomycin reached the primary endpoint of decrease in alkaline phosphatase at 12 weeks[72]. Moreover, addition of metronidazole to ursodeoxycholic acid (UDCA) showed some beneficial effects in biochemical test results and liver histology[73]. The "leaky gut" hypothesis suggests that increased intestinal permeability may lead to translocation of bacterial metabolites from the gut[74] (**Figure 5**). The liver receives approximately 75% of its blood supply from the splanchnic circulation and is constantly exposed to both beneficial and noxious molecules from the intestinal microbiome[75]. This so-called 'gut-liver axis' is essential for the maintenance of health but may also play an important role in pathogenesis of liver and intestinal diseases[75,76]. This dysbiosis may be associated with mucosal immunity dysregulation by modulating intestinal permeability and altering homing of gut-specific lymphocytes[77]. Recently, evidence for an etiologic role of the intestinal microbiome in PSC has been provided by animal model studies. Multidrug resistance gene 2 knockout ($Mdr2^{-/-}$) mice, a murine model for PSC, exhibited a more severe phenotype when maintained under germ-free conditions.[78] However, NOD.c3c4 mice, a murine model for biliary inflammation, exhibits a less severe phenotype when maintained in germ-free conditions.[79] These contradictory findings probably result from the different murine models used and the different intestinal microbiota of these mice, highlighting the complex interaction between the intestinal microbiota and the liver.

The interaction between microbiota and bile acid (BA) metabolism may also play an important role in the PSC-IBD phenotype. It is well known that there is a reciprocal relation

between the microbiome and BA pool. Reduced BA in the gut (such as in situations of obstructive cholestasis like PSC) leads to bacterial overgrowth and inflammation. In the other way around, BA metabolism is a property of the gut bacteria[80].

Recent evidence supports the existence of BA (dys)metabolism in IBD patients due to impaired microbiota enzymatic activity[81]. One of the contributing factors for the difference in phenotype between PSC-IBD patients and IBD controls could potentially be altered concentration and/or composition of colonic bile acids impacting on gut microbiota and stool BA metabolism.

Gut lymphocyte homing

Activated T lymphocytes from the inflamed and permeable gut may enter the enterohepatic circulation and persist as memory cells that cause hepatic inflammation[82, 83]. Some molecular features, such as chemokines and adhesion molecules, are shared by the liver and intestine and could contribute to lymphocyte binding at both sites[82]. T cells activated in the gut during active inflammatory bowel disease could differentiate into effector cells with the ability to bind to both hepatic and mucosal endothelium. The activation and expansion of these memory cells in the liver could eventually lead to the induction of MAdCAM-1 and CCL25 in the liver, promoting the recruitment of $CCR9^{+}$ $\alpha4\beta7^{+}$ mucosal T cells and the development of inflammation[84]. The enterohepatic circulation of lymphocytes may explain the interaction between the colonic immune system triggered by dysbiotic intestinal microbiota and biliary inflammation. This theory is further supported by the finding of memory T-cells with common clonal origin in both the gut and the liver of patients with PSC-IBD.[85]

Findings such as PSC development after colectomy for IBD, or the development of IBD after OLT for PSC, have led some investigators to suggest that aberrant homing of lymphocytes between the intestine and liver could be involved in the pathogenesis of the PSC-IBD phenotype[82].

Environment

Very little is known about the impact of environment in the pathogenesis of PSC. Smoking has been repeatedly associated with a lower risk for developing PSC, independently of the protective effect of smoking in UC.[86-88] Coffee consumption also seems to be associated with a lower risk for PSC,[86] and one study showed that women with PSC are more likely to have

recurrent urinary tract infections[88]. There is a clear need for further studies exploring the impact of environmental factors in the genesis of disease.

The special phenotype of PSC-IBD

The co-occurrence of PSC with IBD is associated with a distinct IBD phenotype (**Figure 6**).

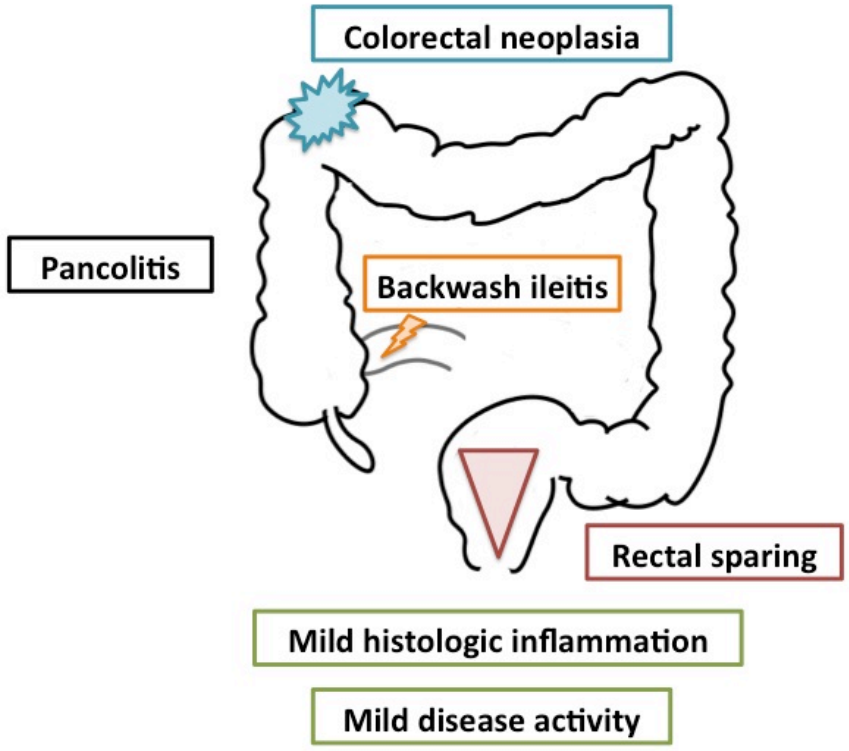


Figure 6 - The special phenotype of PSC-IBD. Patients with PSC-IBD present more often extensive but mild colitis, backwash ileitis, rectal sparing, and right-sided colorectal neoplasia. Reproduced with permission from *Palmela C. et al (2017). Gut Liver. Inflammatory Bowel Disease and Primary Sclerosing Cholangitis: A Review of the Phenotype and Associated Specific Features.* doi: 10.5009/gnl16510. [65].

Both sex and all age groups can be affected, but PSC is more common in men (65.5%), and the mean age of diagnosis is 38.5 ±15.5 years.[56] Patients with PSC-IBD tend to have a PSC diagnosis at a younger age when compared with PSC-alone controls (mean age 33.6 ± 17.2

years versus 58.9 ± 18.2 years; $p < 0.001$)[89]. Some studies indicate that the mean age for IBD diagnosis is higher among PSC-IBD patients compared with IBD controls[64, 65].

The impact of the PSC on the IBD

PSC-IBD patients typically have extensive colonic involvement, irrespective of the IBD sub-type. In a population-based cohort pancolitis was observed in 83% of PSC-UC patients[90], although lower rates have also been reported[65]. In PSC-CD colonic involvement is the most often reported (37-82%), followed by ileo-colic (22-58%), and rarely isolated ileal involvement (2-5%)[58]. Ulcerative proctitis or Crohn's ileitis are very rarely associated with concomitant PSC[90, 91]. The frequency of rectal sparing ranges from 6% to 66% (versus 2-25% in UC without PSC). Backwash ileitis, a mild inflammation occasionally seen in the terminal ileum in patients with UC, has been reported in up to 46% of patients (as compared to 3-24% in UC without PSC)[58].

Despite the higher prevalence of extensive colitis, the intestinal inflammation in PSC-IBD patients is usually quiescent leading to mild symptoms and milder disease course[59, 89]. Typically, the endoscopic and histologic inflammatory activity is highest in the right colon and lowest towards the distal colon[58, 61, 89,92], and on histopathology, the colonic inflammation is mild[59, 93].

There may exist an inverse relationship between PSC disease severity and IBD activity. PSC-IBD patients with more severe liver disease requiring OLT (orthotopic liver transplantation), have less severe UC, with fewer flares and lower steroids and immunosuppressive requirements[94]. In contrast, those not requiring OLT, and therefore with presumably less aggressive liver disease, showed an increased need for intestinal surgery and more frequent colorectal neoplasia[94]. These data are in a certain way supported by a recent study where patients with long-standing IBD were screened with magnetic resonance cholangiopancreatography (MRCP) for PSC[95]. Those with subclinical PSC, were found to have a higher risk of IBD disease progression (with extensive colitis, persistent symptoms and even colectomy). Although not universally confirmed, studies have reported that IBD may worsen after OLT in approximately 30% of patients[53, 96-98]. De-novo IBD after OLT has also been reported and it may develop in 14-30% of PSC patients up to 10 years after transplantation[99].

The impact of IBD on the PSC

The effect of the IBD on the PSC phenotype is less well defined. Combined intrahepatic and extrahepatic biliary involvement has been described to be more common in PSC-IBD patients compared to PSC patients alone (81.5% vs. 46.2%; $p<0.05$)[100], but not universally confirmed[56, 101, 102]. Some studies have suggested that there is an increased prevalence of small-duct PSC in PSC-CD patients as compared to PSC-UC.[103] PSC-UC is more often associated with large-duct PSC as compared to other phenotypes such as small-duct PSC(sdPSC), or PSC associated with auto-immune hepatitis (AIH) (frequency of UC in patients with classical PSC: 58.1% vs 33.5% in sdPSC, and vs 47.7% in PSC-AIH; $P < 0.001$ for both comparisons)[56].

Conflicting data exists on the impact of concomitant IBD on liver-related outcomes[91, 104-106]; however, a recent large multicentric study showed that PSC-UC is associated with a greater risk of progressing to OLT or death by 56% in comparison to PSC-CD and by 15% in comparison to PSC-alone. It has been postulated that the more benign phenotype of PSC-CD may be explained by the increased prevalence of sdPSC. However, in a retrospective study, even large duct PSC-CD patients had less liver-related morbidity and mortality as compared to PSC-UC patients and PSC-alone[103, 107].

Patients in whom colectomy occurs before PSC is diagnosed may have a lower risk of OLT or death (HR 0.71, 95%CI 0.53-0.95), as opposed to those with colon *in situ* at the time of PSC diagnosis[108]. Additionally, several publications have suggested that an intact colon at the time of liver transplant is a strong predictor of PSC recurrence in the allograft[109-111], although not universally confirmed[112].

Altogether these data suggest that PSC severity may have a 'protective' effect on UC´s activity, and on the other hand, that colonic disease may have the opposite effect in the liver disease.

Increased colorectal dysplasia and cancer in PSC-IBD patients

Since its initial description by Broomé *et al.*[113], plenty of studies have now confirmed that patients with PSC-IBD have an increased risk of colorectal neoplasia (CRN, colorectal dysplasia and colorectal cancer)[114-122]. A large meta-analysis evaluating 13,379 patients with IBD, 1,022 (7.63%) of whom had concomitant PSC, showed that there was a three-fold increased risk of CRN and cancer among patients with PSC-IBD compared to the IBD-only

population (odds ratio (OR) 3.24 [95% CI 2.14-4.90])[123]. This trend persisted even after evaluating colorectal cancer (CRC) risk alone (OR 3.41 [95% CI 2.13-5.48]). In a subgroup analysis, PSC-UC patients were found to have a higher risk of both dysplasia (OR 2.98 [95% CI 1.54-5.76]) and cancer (OR 3.01 [95% CI 1.44-6.29]) compared to UC-only patients, although there was high heterogeneity among the studies. Particularly, the PSC-CD population had a non-statistically significant higher risk of CRN and cancer (OR 2.32, p=0.133 and OR 2.91, p=0.388 respectively). Interestingly, in one large cohort describing the risk of cancer in PSC patients, CRN risk was only increased when IBD was also present[124].

All major guidelines consider PSC-IBD patients to be a group for high risk of developing aCRN and thus, routine endoscopic surveillance (preferably using chromoendoscopy) every year, starting from the moment PSC is diagnosed, is strongly advised[7, 125].

Increased hepatobiliary malignancy

PSC is associated with an increased risk of hepatobiliary malignancy, especially of cholangiocarcinoma (CCA).[126] Risk estimations for CCA vary, but highest estimates reach up to a 20% cumulative 30-year risk for PSC patients[56, 127], while the risk of hepatocellular carcinoma or gallbladder carcinoma is far lower.[56] Some centers perform annual imaging studies (either MRCP or ultrasound) together with a serum CA 19-9 for the early detection of CCA,[126, 128] although there are no evidence-based recommendations.[129] When a suspicion of CCA is raised, ERCP with ductal sampling (brush cytology or endobiliary biopsies) is recommended. Although the increased risk of increased CCA risk is independent of concurrent IBD diagnosis, work has shown that prolonged duration of IBD may be associated with a further increased risk.[130] Furthermore, in a large cohort, patients with PSC-UC had a 45% and 35% higher risk of developing hepatobiliary malignancy as compared to PSC-CD and PSC-alone, respectively[56].

The management of PSC

Despite all the advances in the knowledge about disease pathogenesis and management of IBD, PSC remains a disease with a dismal prognosis due to the absence of effective medical therapies, and the progressive nature of disease often resulting in liver cirrhosis and eventually end-stage liver disease[53], requiring orthotopic liver transplantation (OLT)[53]. OLT

or death usually take place within 13.2-21.3 years after the initial diagnosis of PSC, depending on severity of disease.[131]

Patients with PSC-IBD should be managed according to the general IBD guidelines. However, as noted earlier, there are several differences in the IBD disease phenotype of PSC patients (pancolitis, rectal sparing, mild symptoms) that may lead to different management decisions. Close articulation with a specialized hepatologist is warranted when considering treatment options for PSC, such as *UDCA*. When somewhere in the course of PSC-IBD a colectomy is necessary, both an ileal-pouch anal-anastomosis (IPAA) or proctocolectomy and ileostomy can be performed. In case of a colectomy with ileostomy, there is a risk of parastomal varices. In addition, this procedure often results in a rectal remnant remaining in situ, which is at risk of developing rectal stump cancer.[132] Therefore, endoscopic rectal stump surveillance should be performed. There is a higher risk of developing pouchitis after IPAA, affecting 14-90% of cases (versus 33% in patients with conventional IBD)[58, 64, 65, 133]. Furthermore, there may be an increased risk of developing pouch dysplasia in PSC-IBD patients[134]. Nonetheless, the incidence of pouch failure in PSC-IBD seems to be similar to IBD-alone patients[58, 135].

The therapeutic approach to PSC is the same, whether there is concomitant IBD or not. Unfortunately, there is currently insufficient evidence to show differences in effectiveness measures such as mortality, health-related quality of life, cirrhosis, or liver transplantation between any active pharmacological intervention and no intervention[136].

Ursodeoxycolic acid (UDCA) is used extensively, leading to an improvement in liver biochemistry results, but not on important liver-related outcomes.[137] Experimental and animal-model studies have suggested a possible suppressive effect of UDCA on colonic tumor formation[138]. Results on a possible chemopreventive effect of UDCA are conflicting and most studies did not incorporate findings on dosage and treatment duration. A recent meta-analysis found a significant chemopreventive effect of UDCA on the risk of aCRN.[139] Specifically, the risk of all colorectal neoplasia was decreased for low-dose (8-15 mg/kg/day) UDCA use. Notably, a recent study found an increased risk of CRC for patients treated with high doses of UDCA (28–30 mg/kg/day).[140] While high-dose UDCA as a chemoprotective agent or as a maintenance treatment in PSC is discouraged, many practitioners will still contemplate its use in lower doses (20 mg/Kg) albeit further evidence is required[141].

Vedolizumab, a biologic drug approved for the treatment of IBD, blocks gut leukocyte trafficking by preventing the α4ß7 subunit from binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Aberrant expression of these gut adhesion molecules in PSC has opened the possibility of exploring vedolizumab as a potential therapy[142], although no RCT results are available and preliminary results don´t look positive.

The potential role of microbiota in disease pathogenesis is supported by positive results from trials exploring the use of antibiotics in the treatment of PSC. Vancomycin, particularly, has shown promising results[72], and further trials are ongoing.

It is evident that there is an urgent need exists to identify an effective medical treatment for primary sclerosing cholangitis through well-designed RCTs with adequate follow-up that aim to identify differences in outcomes important to people with primary sclerosing cholangitis[136].

Liver transplantation is the only curative option for patients with PSC, although PSC can recur in roughly 25% of the transplanted patients.[143] Although not unanimously shown, a range of studies suggests a persistence or increase in IBD activity after OLT.[144-146] Unfortunately, the evidence on colonic neoplasia risk after transplantation is scarce. While early reports may have suggested an increase in CRC risk post-OLT, more recent evidence did not find such an effect.[147-150] Interestingly, a recent study comparing dysplasia progression rates between transplanted and non-transplanted patients found a longer time to progression of LGD in patients who had been transplanted.[151]

RATIONAL TO CONDUCT RESEARCH PRESENTED IN THIS THESIS

There are multiple aspects of PSC-IBD that remain to be solved. Despite all the advances in the knowledge about disease pathogenesis and management of IBD, the special PSC-IBD phenotype remains unexplored and in that sense, PSC can be considered an orphan disease. Investigators have speculated about possible alterations in the concentration and/or composition of colonic bile acids (BA), especially as a justification for the increased risk of CRN[118]. Notably, patients with PSC alone, without IBD do not have an increased risk of developing CRN[124], suggesting that additional factors besides BA must be involved. The recent discovery of receptors implicated in BA homeostasis and their correlation with gut inflammation and carcinogenesis, as well advances in our knowledge about intestinal microflora, offer new insights into this subject.

BA homeostasis is tightly regulated by the activation of Farnesoid X Receptor (FXR), a nuclear BA receptor, expressed at high levels in liver and intestine [152]. BA act as ligands for FRX, which in turn serves as a biological sensor for BA [153]. The interaction between BA and FXR has been shown to play a key role not only in the enterohepatic circulation, but also in regulation of inflammatory liver and intestinal responses, intestinal barrier function and antibacterial defense [154]. Additionally, this bile acid receptor has also been shown to be involved in colorectal cancer.

The consequences of cholestasis and of a BA pool altered by the diverse bacteria in the inflamed colon are not fully known, but we can hypothesize that a specific alteration of the microbiome in PSC-IBD patients could participate in the special phenotype, including in colorectal carcinogenesis either directly or through BA transformation. Indeed, several lines of evidence suggest a role for the gut microbiome in the pathogenesis of PSC associated with IBD. Alternatively, changes in BA composition due to the liver disease could also participate and lead to the selection anti-inflammatory or more pro-carcinogenic strains of bacteria in the gut, that could be associated with this phenotype.

Herein we plan to explore the interactions between gut dysbiosis, BA metabolism, and expression of BA receptors in the inflamed colon that may contribute to the special phenotype observed in PSC-IBD patients. Additionally, we plan to further explore clinical aspects involved in the management of this special population, for which the current

literature does not provide and answer, specifically the management and follow-up of low-grade dysplasia in PSC-IBD.

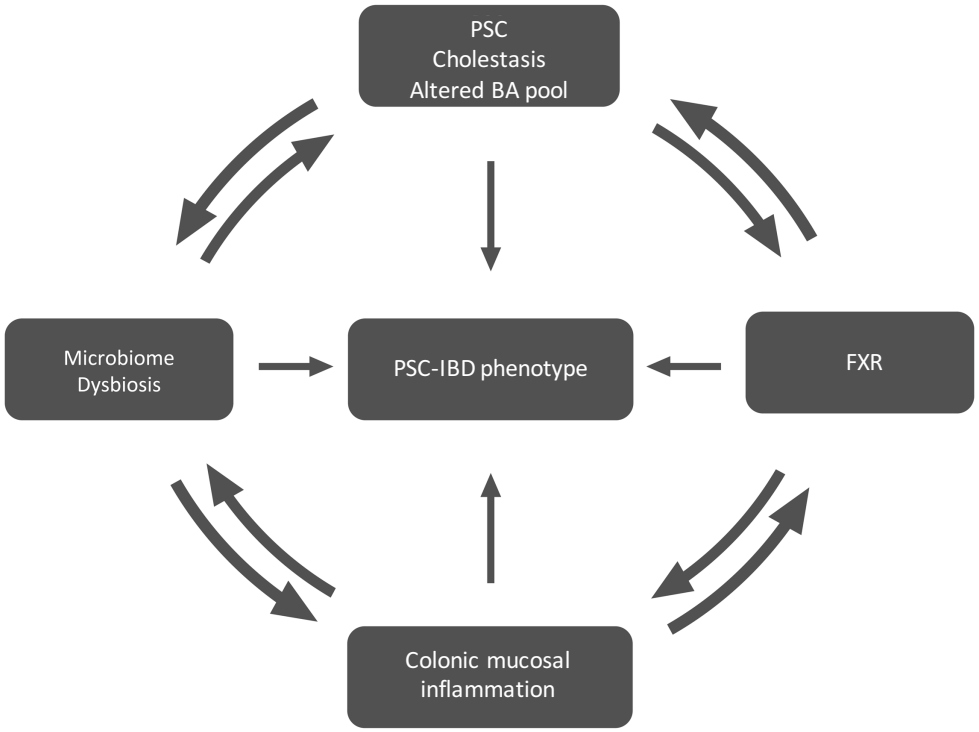


Figure 7 - Proposed interaction between colonic inflammation, microbiome and altered bile acid pool and bile acid metabolism through FXR, that could explain the special phenotype primary sclerosing cholangitis and inflammatory bowel disease (PSC-IBD). *Adapted from Torres J et al. (2011). APT 2011. Review article: colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease. doi: 10.1111/j.1365-2036.2011.04753*[155]

PSC associated with IBD represents, therefore, a unique model in which to study bile acid metabolism, inflammation and interactions with gut flora. A better understanding of these processes might provide new insights into the pathogenic mechanisms involved in this specific phenotype (**Figure 7**).

CHAPTER 2

THE ROLE OF BILE ACID RECEPTORS IN THE SPECIAL PSC-IBD PHENOTYPE AND ITS RELATION WITH COLORECTAL NEOPLASIA

INTRODUCTION

Bile acids (BA) are important signaling molecules, acting in inflammation and metabolism, through activation of BA receptors such as the nuclear BA receptor Farnesoid X receptor (FXR).

FXR, is a nuclear receptor, recently discovered and characterised[156]. FXR is the main BA receptor, regulating virtually every aspect of bile acids metabolism[157], the entero-hepatic circulation and the cross-talk between the liver and colon (**Figure 8**)[158].

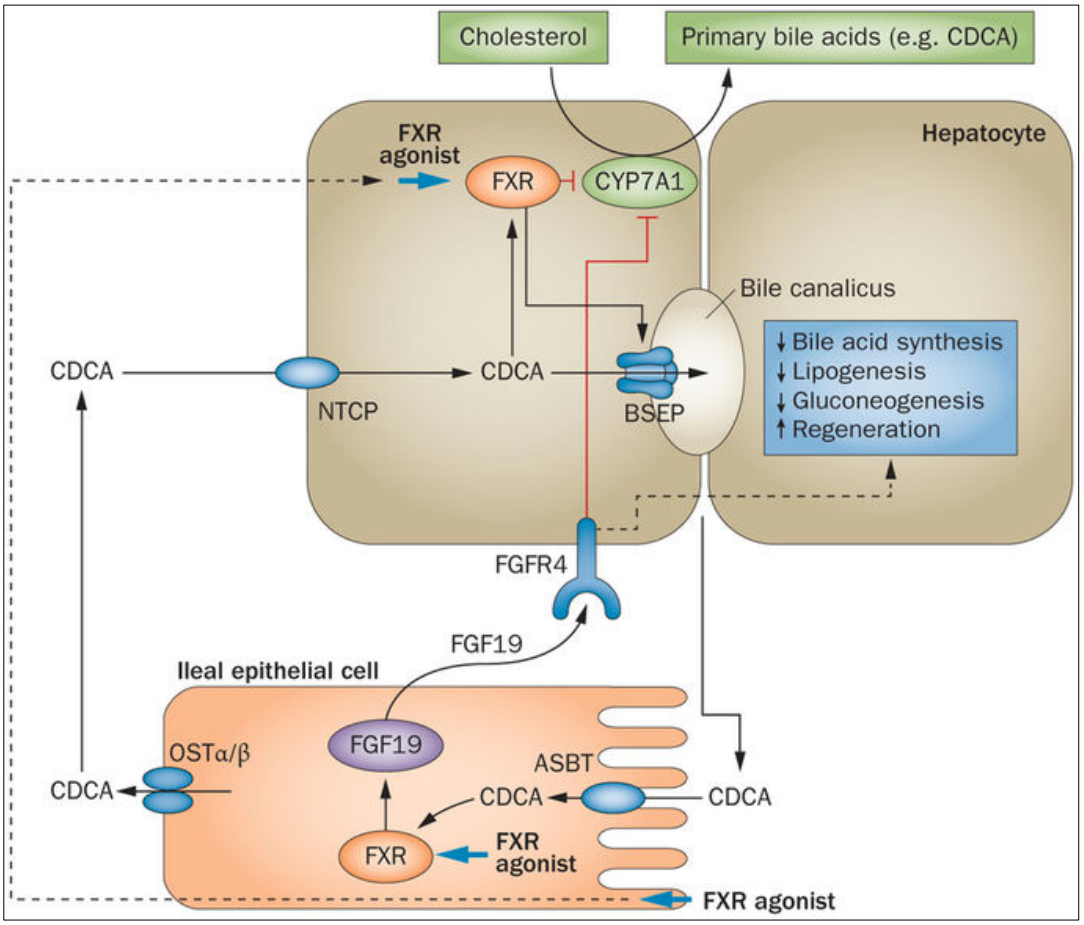


Figure 8 - Illustration of the role of FXR in entero-hepatic circulation. Primary Bile acids [chenodeoxycholic acid (CDCA) and cholic acid (CA)] are produced in the liver by the conversion of cholesterol into primary bile acids, a reaction mediated by the enzyme cholesterol 7-α-monooxygenase (CYP7A1), and then conjugated with glycine or taurine becoming water-soluble. After a meal, bile containing bile acids (BA) is secreted into the intestine. Normally, most of the BA secreted by the liver are efficiently reabsorbed in the terminal ileum, through an active process carried out by the apical sodium-dependent BA transporter (ASBT), leaving only approximately 5% of the total BA to reach the colonic lumen. In the colon, mostly on the right side, primary BA are

transformed into secondary BA by bacterially-mediated processes. In the terminal ileum FXR induces synthesis of fibroblast growth factor-19, which is then secreted into the portal circulation. In the liver, FGF19 binds to its receptor, fibroblast growth factor receptor 4 (FGFR4), which will eventually signal to the liver the end of the entero-hepatic circulation and inhibit the *CYP7A1*, thus downregulating bile acid synthesis[159]. *Reproduced with permission from Schaap, F. G. et al. (2013) Bile acid receptors as targets for drug development Nat. Rev. Gastroenterol. Hepatol. doi:10.1038/nrgastro.2013*[158].

It is expressed at high levels in liver and intestine, especially in the terminal ileum and proximal colon[152]. In enterocytes, BA-dependent FXR activation results mainly in two events. First, FXR induces synthesis of fibroblast growth factor-19 (FGF19), which is then secreted into the portal circulation and acts on hepatocytes to suppress the enzyme cholesterol 7-α-monooxygenase (CYP7A1), the rate-limiting enzyme responsible for BA synthesis[160]. Second, FXR-activation increases expression of ileal BA-binding protein (IBABP) and basolateral organic solute transporter (OST) and is coupled to reduced apical sodium-dependent BA transporter (ASBT) expression, resulting in decreased BA intestinal absorption and prevention of intracellular BA accumulation[161]. Therefore, FXR-mediated mechanisms prevent the noxious effects of BA accumulation on hepatocytes and on the cells lining the intestinal and biliary tract[154], playing a key role not only in the enterohepatic circulation, but also in the regulation of inflammatory responses in the liver and intestine[154]. Several work has demonstrated that besides its role on bile acid and lipid metabolism, this receptor is also involved in mucosal immune response. FXR exerts a number of modulatory and immune-regulatory effects on ileal and colonic epithelial cell lines[162]:

1. <u>FXR has anti-inflammatory properties</u>

FXR is expressed in macrophages, and its activation results in the robust down-regulation of the expression of several IFNγ regulated genes and several inflammatory signaling pathways, such as the STAT3 (signal transducers and activators of transcription 3) pathway; however, in the setting on inflammation, FXR is down-regulated through IFNγ (Interferon gamma) mediated repression mediated by STAT1 activation[163]. FXR-knockout mice are more susceptible to a model of chronic induced intestinal inflammation and present increased chemically-induced [trinitrobenzensulfonic acid (TNBS) or dextrane sodium sulfate (DSS) models)] colitis severity; conversely, the administration of an FXR agonist prevents DSS- and TNBS-induced intestinal inflammation. This is corroborated by human

data showing that colonic inflammation down-regulates colonic FXR-expression, since patients with IBD present lower expression of this receptor in areas of inflamed mucosa[164], and FXR mRNA expression was almost undetectable in colon biopsies from macroscopically-inflamed areas in patients with Crohn's disease[164].

2. FXR has a role in barrier function by regulating antibacterial growth

FXR knockout mice (FXR-/-) mice display dysregulated immune response, compromised epithelial barrier, increased intestinal permeability, and increased levels of bacteria in the ileum and mesenteric lymph nodes. Conversely, in a model of bile duct ligation (a model of obstructive cholestasis similar to PSC), the resulting small intestine bacterial overgrowth, can be reversed by FXR agonist or by administering BA that leads to FXR activation[165].

3. FXR may be involved in colorectal carcinogenesis

FXR has an important role in maintaining BA concentration, thereby preventing BA-cytotoxicity. FXR-knockout mice have an increased colorectal carcinogenesis (increased number of tumors and tumors size) in murine intestine tumorigenesis models: APC^{min} mice (adenomatous polyposis coli) and azoxymethane (AOM)-induced colon cancer[166], through increased cell proliferation via promotion of *Wnt* signaling, and up-regulation in the expression of genes involved in cell cycle progression and inflammation, such as cyclin D1 and interleukin-6[166]. In humans, it has been shown that FXR is down-regulated with a reciprocal relationship between the degree of expression and tumor stage[167, 168].

These data suggest that in PSC-IBD patients, colonic inflammation, in part by inactivating FXR-mediated mechanisms, could exacerbate the toxic effects of secondary BA on colonic cells. The involvement of the FXR receptor in both intestinal inflammation and carcinogenesis makes it an interesting target to study in colitis as well in colitis-associated neoplasia. Furthermore, being the major regulator of the entero-hepatic circulation of bile acids, the influence of concomitant PSC in FXR's colonic expression is of interest to study.

RATIONAL AND AIMS

Rational: The expression of FXR has been reported to be downregulated in the setting of colonic inflammation[169, 170] and in colon adenomas and cancers of the colon[162]. However, practically nothing is known about the expression of FXR in the setting of chronic

inflammation of the colon, such as UC, and moreover in PSC-IBD; specifically, nothing is known about the expression of FXR with respect to the *degree* of inflammation (active vs. quiescent; mild versus severe), the *location* in the colon, or whether there might be differences between IBD and PSC-IBD patients. Likewise, this receptor has never been studied in the setting of colitis-associated neoplasia (dysplasia or cancers). We hypothesized that the expression of FXR would be lower in mucosa that is involved with active inflammation, but not in mucosa with only quiescent inflammation and that FXR´s expression would be lower in mucosa that is involved with dysplasia and cancer compared to background mucosa with quiescent inflammation.

Aim: To analyze the expression of the nuclear receptor FXR in non-neoplastic and neoplastic tissue of patients with IBD with, and without, PSC.

Approach: Patients with UC, and PSC-IBD were identified from the UC-Surveillance Database, and from the GI Pathology database, at Mount Sinai Hospital, New York. Archived paraffin embedded tissues taken during surveillance colonoscopy were retrieved, sectioned and stained for FXR by immunohistochemistry. Expression was correlated with degree of inflammation, degree of dysplasia, location in the colon and disease state.

Contribution of the PhD candidate:

The candidate had the idea to study this receptor in biopsies from PSC-IBD and IBD alone patients. She wrote the protocol, selected samples from the pathology repository at Mount Sinai and performed the immunohistochemistry in collaboration with Xiuliang Bao at Mount Sinai. She had the idea to study methylation in cancer cell lines in relation to FXR, which was performed by Xiuliang Bao. She read the slides in collaboration with Professor Noam Harpaz, an expert GI pathologist and Dr. Alina Iuga, also a GI pathologist at Mount Sinai. The PhD candidate performed the statistical analysis for the project, presented the work in international meetings and finally wrote the paper that was published in Inflammatory Bowel Diseases (**Impact factor: 4.5**): *"**Farnesoid X receptor expression is decreased in colonic mucosa of patients with primary sclerosing cholangitis and colitis-associated neoplasia**. Torres J, Bao X, Iuga AC, Chen A, Harpaz N, Ullman T, Cohen BL, Pineton de Chambrun G, Asciutti S, Odin JA, Sachar DB, Gaskins HR, Setchell K, Colombel JF, Itzkowitz SH. Inflamm Bowel Dis. 2013 Feb;19(2):275-82. doi: 10.1097/MIB.0b013e318286ff2e."*

MATERIAL AND METHODS

Case Selection

Following approval by the Mount Sinai School of Medicine Institutional Review Board, patients were identified using the Mount Sinai Gastrointestinal Pathology and UC-Surveillance databases described in previous studies[171]. Besides demographic information, clinical information and pathologic findings from colonoscopies and operations, including anatomic extent of disease at diagnosis, presence and grade of any dysplasia, and presence and severity of inflammation at each biopsied segment of colon, were recorded. Additionally, for the measurement of FXR expression in colitis-associated neoplasms, we analyzed samples in two tissue microarrays (TMAs) containing samples of colorectal neoplasia (CRN) from IBD patients (colorectal cancer, low and high-grade dysplasia).

Patients with extensive UC at diagnosis, with or without PSC, were identified and their pathology blocks retrieved. The presence or absence of PSC was based on the results of serum biochemical tests and cholangiography and/or hepatic biopsy. Patients with Crohn's disease and indeterminate IBD were excluded. From each patient, whenever available, samples from the right colon (RC) and left colon (LC) were recovered. An attempt was made to have samples representative of different degrees of histological inflammation.

One hundred and twelve patients (84 males, median age 51y) were included in this study. There were 24 patients with concomitant PSC. All patients had extensive UC at diagnosis (defined by greatest microscopic extent proximal to the splenic flexure). Median disease duration for the patients in whom that information was available was 20±13 years (range 1-60y). From all these patients, 231 colonic samples (biopsy specimens, surgical samples, and tissue microarrays) were obtained: 155 from UC patients (62 from the RC) and 76 from PSC-UC (39 from the RC). From 9 PSC-UC and 62 UC patients, 79 samples of CRN (19 colonoscopy biopsies and 60 samples from the TMAs) were analyzed. (**Figure 9**).

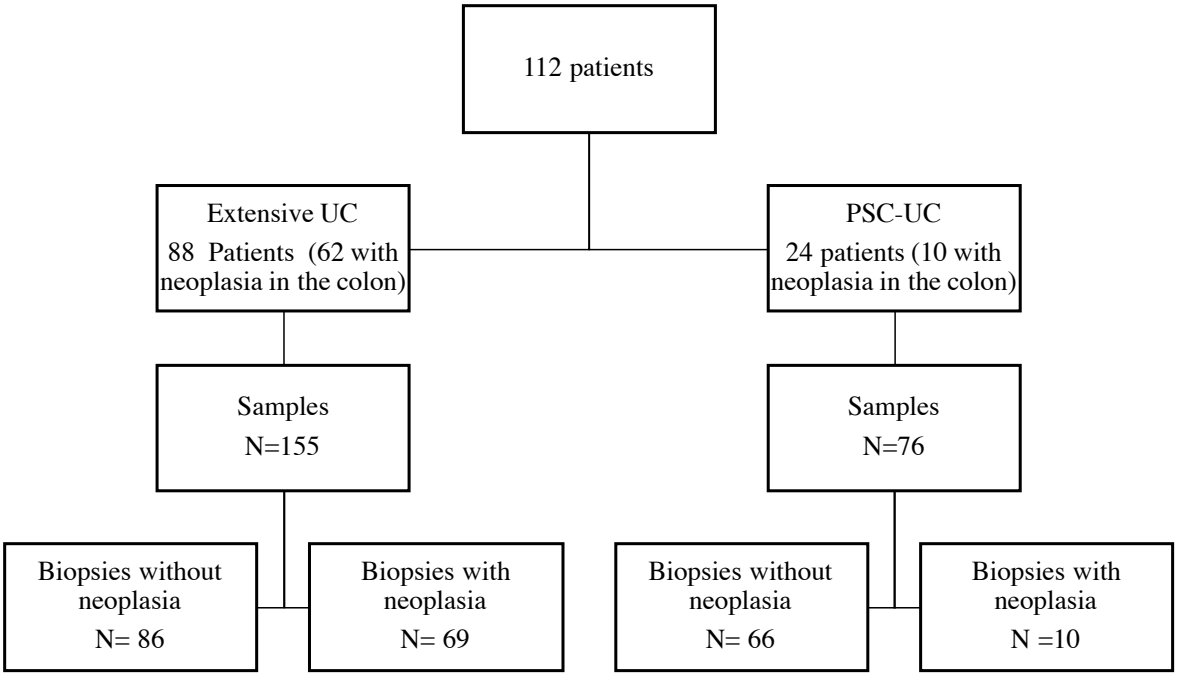


Figure 9 – Distribution of patients and samples per group. Samples correspond to either biopsies, tissue microarrays or surgical specimens.

Histologic grading of inflammation and neoplasia

For each non-dysplastic sample, the severity of histologic inflammation was taken from the pathology report. These specimens had been scored according to the histologic activity index (HAI) that we have previously validated and described[171]. The histological criteria for grading severity of inflammation was developed by one of the authors (N.H.) and placed into routine use at The Mount Sinai Hospital in 1988. These criteria have remained unchanged since their introduction. All reports had been issued by one of a small group of gastrointestinal pathologists, trained by N.H., using the narrative equivalent of a standardized histological activity index (HAI)[172]. The degree of inflammation for each biopsy site was scored as follows: (0) inactive/absent, (1) mild, (2) moderate or (3) severe[171]. An HAI of 0 (inactive) corresponds to inactive colitis with no cryptitis or crypt abscesses; an HAI of 1 (mild) corresponds to mildly active colitis with one crypt abscess; an HAI of 2 (moderate) corresponds to moderately active colitis with cryptitis involving >50% of crypts; and a HAI of 3 (severe) corresponds to severely active colitis with ulceration[171].

Dysplasia was diagnosed initially as log-grade or high-grade dysplasia, by one of the authors (N.H.) using the criteria of the IBD Dysplasia Morphology Study Group[173] and confirmed as a part of this study by a second GI pathologist (A.I.).

Immunohistochemistry

Immunohistochemistry for FXR was performed manually on samples and tissue microarrays (TMAs), using a mouse anti-human FXR monoclonal antibody (Perseus Proteomics, Tokyo, Japan). This antibody specifically recognizes human FXR and cross-reacts with mouse and rat FXR. From the formalin-fixed, paraffin-embedded tissue blocks of biopsies and colectomy specimens, sequential sections were cut at 4-μm thickness and mounted on adhesive slides. Slides were de-parafinnized in xylene and subsequently washed in graded ethanol (100%, followed by 95%) and re-hydrated in distilled water. For antigen retrieval, sections were incubated in a microwave for 30 minutes using a 0.1% sodium citrate buffer and subsequently washed in PBS at room temperature. Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 for 10 minutes and then rinsed three times with PBS (Phosphate Buffered Saline**)**. Sections were incubated for 60 minutes at room temperature in 2% BSA (bovine serum albumin) to avoid nonspecific signal, and then overnight at 4^{o}C with the primary anti-FXR antibody. Subsequently, slides were rinsed 3 times in PBS and treated for 30 min at room temperature with a polyclonal anti-mouse secondary antibody (EnVision+ System-HRP Labelled Polymer Anti-mouse - Dako, Denmark), and again washed three times with PBS. Slides were then incubated with diaminobenzidine (DAB) using the Peroxidase substrate DAB kit ^{TM} (Vector Laboratories). After cleansing with water, slides were counterstained with Harris modified Hematoxylin solution for 50 seconds, dipped in ethanol and in ammonia water, with rinse in tap water in between. Finally, sections were de-hydrated in 95% and 100% alcohol consecutively, washed with xylene and mounted with VectaMount ^{TM} (Vector Laboratories). At least one section with normal small intestinal mucosa was included for each run as a positive control.

Evaluation of immunohistochemistry

FXR nuclear expression was scored by two independent, experienced observers (S.I. and A.I.) who were blinded to the clinic-pathological information. FXR nuclear staining intensity

was scored as absent (0), weak (+) and strong (++). There was a 95% concordance between the two observers. Differences were resolved by consensus evaluation of the slides.

FXR gene de-methylation experiments

Cell Culture:

Human liver cell line HepG-2 cells were cultured in RPMI-1640 medium (Invitrogen, CA) with 10% FBS (fetal bovine serum) Cell at 37°C in 5% CO_2 atmosphere. Human colorectal cancer cell lines were cultured in DMEM (Dulbecco's Modified Eagle's medium) (Invitrogen, CA) with 10% FBS Cell at 37°C in 5% CO2 atmosphere. HepG-2, Caco-2, HCT-116, HT-2 and SW-480 cells were seeded at a concentration $1x10^4$ cells/ml in 6-well cell culture plate, 2 ml/well and the following day, were treated with 0, 1 and 10 μM 5-Aza-2'-deooxycytidine (5-Aza) (Sigma-Aldrich, MO) in fresh culture medium. 5-Aza was removed by replacing the medium 24 hours later. The cells were harvested 4 days after removal of 5-Aza for RNA extraction.

Quantitative real –time PCR analysis of FXR mRNA expression:

Total RNA was extracted using RNeasy Mini Kit (Qiagen, CA). One μg total RNA was used for cDNA synthesis in RNA to DNA EcoDry Premix (Double Primed) (Clontech, CA). FXR mRNA levels were evaluated by SYBE Green assay using advantage qPCR premix (Clontech, CA) and primers: 5'-GCCTGTCTCCTGGGTCGCCT-3' (forward) and 5'-TCCCCATCACTGCACGTCCCA-3' located in exon 11. The mRNA level of the target genes was normalized to GAPDH mRNA. Primers of GAPDH as: 5'-ACCACAGTCCATGCCATCAC-3' and 5'- TCCACCACCCTGTTGCTGTA-3'. All samples were analyzed in triplicate.

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences - SPSS for Mac (version 19.0). When appropriate, Fischer's exact test, Student's t and χ^2 tests were used for comparison between groups. Statistical significance was set at $p<0.05$.

RESULTS

FXR expression in non-neoplastic mucosa

There were 152 samples without dysplasia from 62 patients: 66 from 22 PSC-UC patients, and 86 from 40 UC patients. Among the non-dysplastic samples, 70 were from 21 patients who had no neoplasia in the colon at the time of sampling or surgery; 82 samples were from patients that displayed with neoplasia elsewhere in the colon. Seventy-two samples were from the RC (mostly ascending colon and proximal transverse) and 80 from the LC (mostly sigmoid colon).

We observed that FXR expression was higher in the RC than the LC. Overall, 32% of the samples from the RC demonstrated strong expression, compared to only 14% in the LC (p= 0.011). When stratified by disease type, this difference remained statistically significant only for UC samples: 39.5% of the samples from the RC versus 16.7% had strong FXR expression (p= 0.017). In PSC-UC samples, while there was also a trend towards stronger FXR expression in the proximal colon (strong FXR expression in 23.5% of samples from RC versus 9.4% from LC), this did not reach significance (p= 0.11). Thus, FXR expression seems to be weaker in the left colon of patients with UC and PSC-UC. FXR expression did not differ according to location or disease type in samples from patients without neoplasia compared to those with neoplasia somewhere in the colon (data not shown).

When we compared FXR expression to the degree of inflammation, samples with the highest degrees of inflammation (moderate or severe inflammation) demonstrated FXR expression that was almost always absent or weak in both UC and PSC-UC, regardless of colonic location (**Table 1**).

Inflammation	Location	Disease type (n)	Absent or Weak FXR Expression n (%)	Strong FXR Expression n (%)	P value
Quiescent-mild	RC	PSC-UC (29)	22 (75.9)	7 (24.1)	0.017
		UC (25)	11 (44)	14 (56)	

	LC	PSC-UC (29)	26 (89.7)	3 (10.3)	0.52
		UC (30)	26 (86.7)	4 (13.3)	
Moderate-severe	RC	PSC-UC (5)	4 (80)	1 (20)	0.49
		UC (13)	12 (92.3)	1 (7.7)	
	LC	PSC-UC (3)	3 (100)	0 (0)	0.51
		UC (18)	14 (77.8)	4 (22.2)	

Table 1 - Relationship between degree of FXR expression and the degree of inflammation according to colonic location and disease type.

Very few of the highly-inflamed samples demonstrated strong FXR expression. With quiescent or mild inflammation, FXR expression was almost always absent or weak in the LC (87% in UC, 90% in PSC-UC), reflecting the normal distal decrease in FXR expression (**Figure 10, E and F**). In samples from the RC of UC patients, FXR expression decreased with higher degrees of inflammation: 56% of specimens with quiescent/mild inflammation had strong FXR expression, compared to 7.7% (1/13) with moderate/severe inflammation (p=0.005). Unexpectedly, the same pattern of FXR expression was not observed in the RC of PSC-UC patients: 24% of the RC samples displaying quiescent-mild inflammation had strong FXR expression compared to 20% of samples with moderate/severe inflammation (**Figure 10, B and C**). Indeed, the main difference between PSC-UC and UC was the finding that in the RC, with quiescent or mild inflammation 56% of UC samples retained strong FXR expression, compared to only 24% of PSC-UC samples (p= 0.017) (**Table 1; Figure 10**).

Similar to previous observations in normal colon[174], we observed that FXR immunoreactivity presented a gradient of expression throughout the crypts, which was especially evident in the RC. Thus, FXR expression was stronger at the crypt surface and gradually decreased towards the base of the crypts where it was often absent (see **Figure 10 B**).

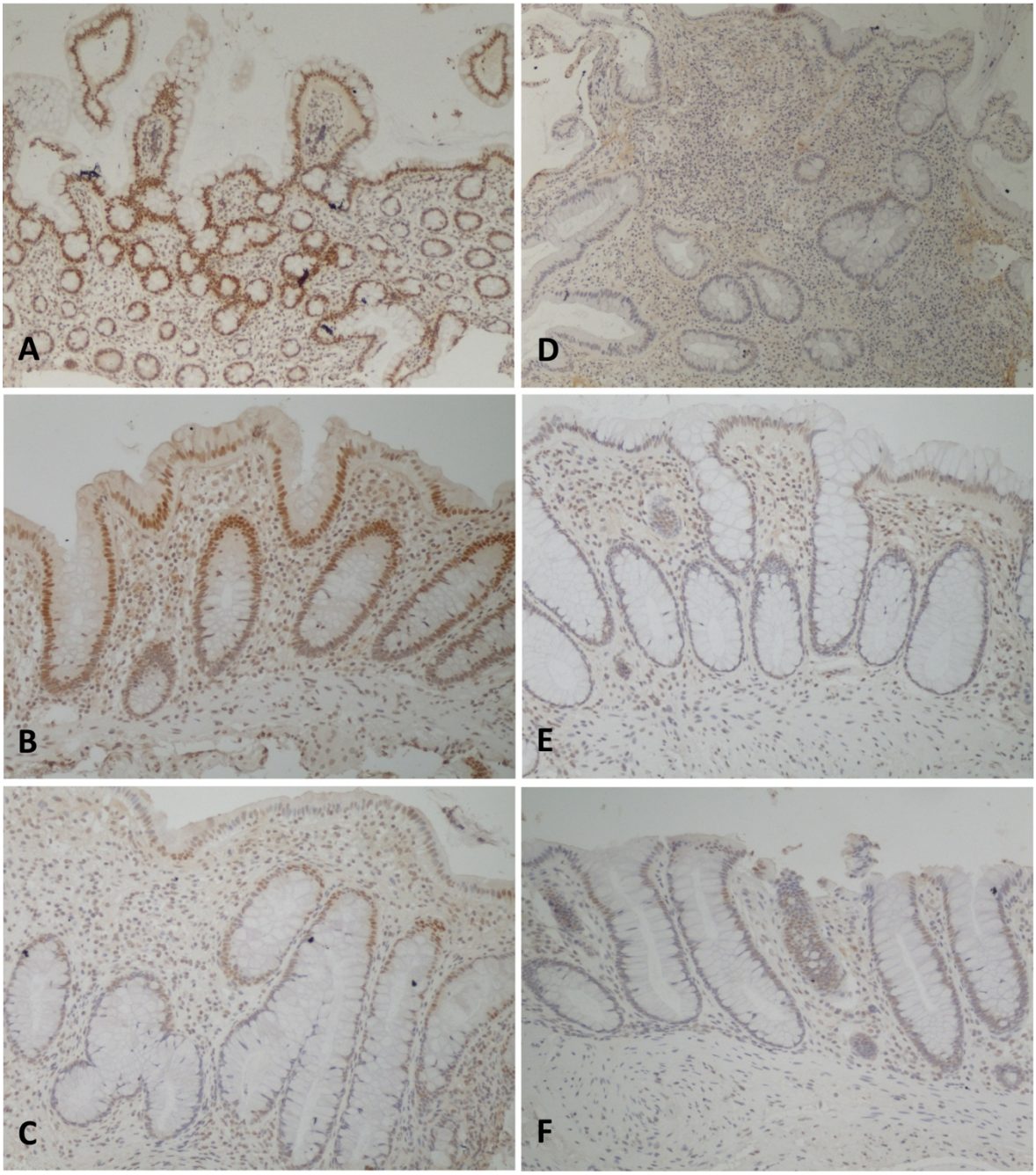


Figure 10 – FXR immunohistochemical staining of non-neoplastic mucosa. A) Normal terminal ileum from patient with UC: there is strong nuclear staining in villi with gradual decrease towards the crypts (10x) B) RC biopsy of a patient with UC and quiescent inflammation; a strong nuclear positivity at the surface and upper part of the colonic glands with a gradual loss of expression in the crypts is seen (20x). C) RC biopsy of patient with PSC and quiescent inflammation displaying diminished FXR nuclear expression (20x). D) RC of a patient with UC and moderate inflammation: decreased FXR nuclear expression compared to uninflamed mucosa in B (20x). E) Uninflamed LC in a patient with UC lacking strong FXR staining (20x) F) Uninflamed LC of patient with PSC-UC displaying only minimal or no FXR nuclear staining (20x).

FXR expression in neoplastic mucosa

There were 52 colorectal cancers (CRC) (15 well differentiated; 14 moderately differentiated; 23 poorly differentiated), 6 high-grade dysplasia (HGD) and 21 low-grade dysplasia (LGD).

Overall, FXR expression was absent in most (57/79; 72.2%) of the dysplastic and CRC samples. Among the samples maintaining any FXR expression, there was an inverse correlation with the degree of neoplasia (**Figure 11**).

FXR was expressed in LGD (13/21; 61.9%), HGD (3/6; 50%), and CRC (6/52; 11.5%), although the difference was significant only between dysplastic samples versus cancer (**Table 2**).

CRN	Absent FXR expression n (%)	Low FXR expression n (%)	Strong FXR expression n (%)	P value	
				LGD	HGD
LGD (n=21)	8 (38.1%)	13 (61.9%)	0	ns	
HGD (n=6)	3 (50%)	2 (33.3%)	1 (16.7%)		
CRC (n=52)	46 (88.5%)	6 (11.5%)	0	< 0.001	0.003

Table 2 - Relationship between FXR expression and degree of colitis-associated neoplasia; ns: non-significant.

No significant differences were observed according to colonic location of the neoplasm, or between UC and PSC-UC patients (data not shown). In the CRC samples that retained some weak expression, no differences were found according to cancer differentiation.

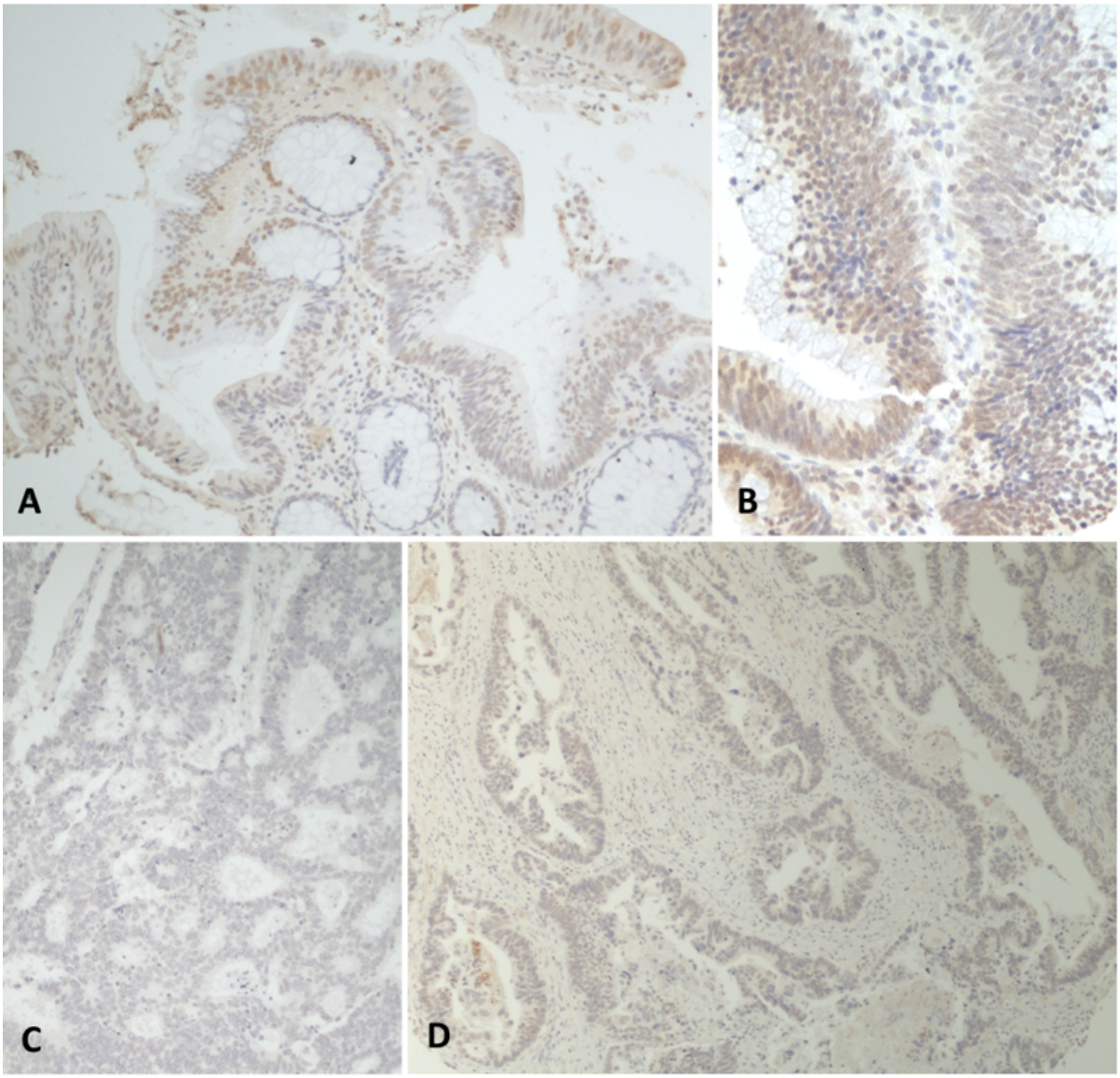


Figure 11 - Examples of FXR staining in colitis-associated neoplasia. A) LGD showing retained FXR nuclear expression (20x) B) High power image of a focus of LGD highlights nuclear FXR positivity (40x) C) HGD: loss of FXR expression (20x). D) Invasive adenocarcinoma: loss of FXR expression (20x).

In Vitro Studies with Colon Carcinoma Cell Lines

Hep2G cell line is a human liver cancer cell line, while Caco-2, SW480, HT-29, and HCT-116 are CRC cell lines. We wanted to assess FXR expression in colon versus liver cancer cell lines, and also to study the potential effect of DNA methylation as mechanism for FXR down-regulation in cancer. Therefore, we treated cancer cell lines, with 5-Azacytidine, a

demethylation agent. We found that, compared to HepG2 cell line, FXR expression at the mRNA level was very low, and was undetectable by western blot in all 4 CRC cell lines. While there were no significant changes in FXR mRNA expression in Caco-2, SW480 and HepG2 cells after treated with 5-aza (**Figure 12**), in HCT-116 and SW480 cells, 5-aza treatment resulted in significant dose-dependent increases in FXR mRNA expression.

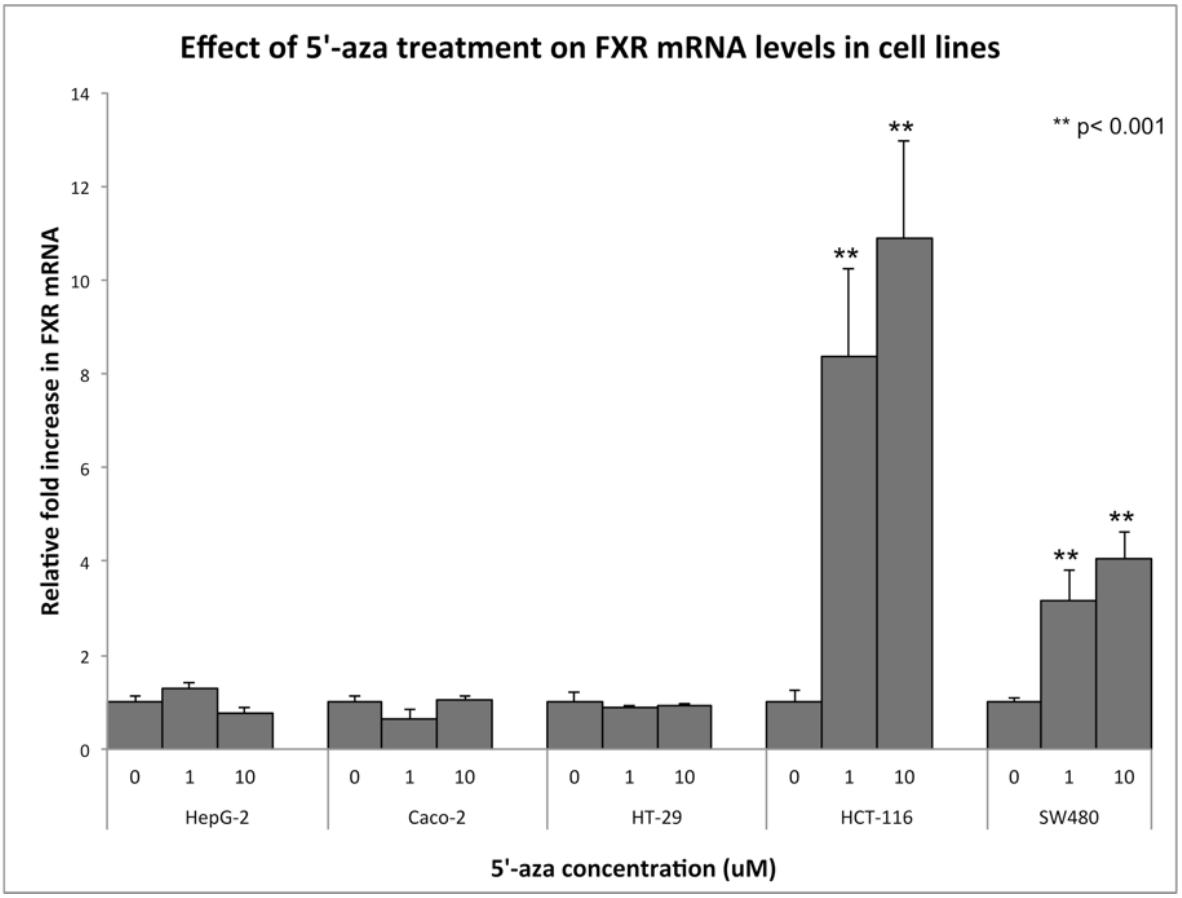


Figure 12 - Effect of 5'aza treatment on FXR mRNA levels in Liver cancer and CRC cell lines.

DISCUSSION

The involvement of the FXR nuclear bile acid receptor in both intestinal inflammation and carcinogenesis makes it an interesting target to study in colitis and in colitis-associated neoplasia. Furthermore, being the major regulator of the entero-hepatic circulation of bile acids, we specifically wanted to analyze the influence of concomitant PSC in FXR's colonic expression.

A recent study showed that there was no difference in ileal and ascending colon FXR mRNA expression between controls, CD, and UC patients in remission[175]. However, the distribution of FXR throughout the colon of patients with UC, or the regional distribution and relation to inflammation in PSC-UC patients had not previously been reported. In UC patients, we observed a distal decrease in FXR expression, with a stronger expression in the RC samples as compared to the LC samples. This pattern of distribution had already been described in normal subjects, and proposed to occur in parallel with the proximal-distal gradient of bile acid flow along the colon[174]. However, when we stratified by disease type, we observed decreased FXR expression in samples from the RC of PSC-UC patients.

Since FXR has been shown to be down-regulated in the presence of intestinal inflammation, we also analyzed results according to the degree of histologically active inflammation. Perhaps not unexpectedly, we observed that with more severe inflammation, FXR expression was usually absent or weak, independent of location or disease type. With quiescent/mild inflammation, FXR expression was also typically weak/absent in both UC and PSC-UC patients if the tissue sample was from the LC, reflecting the normal distal decrease in FXR expression. Interestingly, with little or no inflammation, the RC of UC patients retained the normal pattern of rather high expression of FXR, but the RC of PSC-UC patients did not. Thus, PSC-UC patients appear to lose FXR expression in the RC even without much inflammation.

There are data implicating FXR as a tumor suppressor gene[176]. FXR is decreased in human sporadic CRC with an inverse correlation between the degree of expression and tumor stage [162, 174]. Furthermore, $FXR^{-/-}$ mice have been shown to have increased intestinal carcinogenesis, increased cell proliferation via promotion of *Wnt* signaling and up-regulation in the expression of genes involved in cell cycle progression and inflammation, such as cyclin D1 and interleukin-6[166]. The expression of FXR in the different stages of colitis-associated neoplasia had not yet been described. We herein observed that there was an inverse correlation within the neoplastic sequence in colitis-associated carcinogenesis, with complete loss of FXR expression in 38%, 50%, and 88.5% of LGD, HGD and colitis-associated cancers, respectively. FXR expression in the non-neoplastic adjacent mucosa did not present a similar pattern of expression (data not shown) and therefore, it is likely that FXR down-regulation occurs during neoplastic transformation from LGD, to HGD and finally adenocarcinoma, rather than representing a field defect.

We additionally sought to explore the potential role of DNA methylation as a molecular mechanism of down-regulation of FXR mRNA expression in colorectal cancer cell lines. DNA methylation is associated with histone modifications and the interplay of these epigenetic modifications is crucial to regulate the functioning of the genome by changing chromatin architecture[177]. The covalent addition of a methyl group occurs generally in cytosine within CpG dinucleotides which are concentrated in large clusters called CpG islands in the promoter region of some genes[177]. It is commonly known that inactivation of certain tumor-suppressor genes occurs as a consequence of hypermethylation within the promoter regions. Indeed, DNA methylation is a common mechanism occurring in the development and progression of sporadic CRC, which may also have a role in colitis-associated neoplasia[178]. Putative CpG islands within the promoter and the fourth exon of the human FXR gene have previously been described[179]. We observed that while in Caco-2, HT-29 and HepG2 cell lines there were no changes in FXR mRNA expression after 5-aza treatment, in HCT-116 and SW480 cells, 5-aza treatment resulted in dose-dependent increases in FXR mRNA expression. This suggests that loss of expression of FXR in colonic cells may be regulated, at least in part, by gene silencing by DNA hypermethylation.

Bearing in mind the role of FXR in colorectal carcinogenesis, it is tempting to suggest that FXR' down-regulation observed in PSC-UC samples could explain, in part, the high risk of right-sided CRN in these patients, even in the presence of quiescent to mild inflammation. There are data suggesting that during cholestatic liver disease, such as PSC, intestinal BA absorption is reduced which could lead to a relative increase of BA in the proximal colon, in turn producing heightened conversion of primary bile acids into secondary, more carcinogenic, bile acids [180, 181]. Down-regulation of FXR could therefore expose colonocytes to high levels of secondary bile acids or other toxic products, increasing carcinogenesis risk.

Our study had some limitations. Although a large number of samples were studied overall, the difference of FXR expression in the RC of PSC-UC patients was based on a somewhat small sample size. These findings should be confirmed in other studies. In addition, as a descriptive, retrospective immunohistochemical study on archival tissue, our study does not allow us to draw any mechanistic conclusions, such as whether low FXR expression results from FXR down-regulation or from post-transcriptional events modulating FXR expression. Further research is required to elucidate the underlying mechanisms for FXR down-regulation in PSC-UC patients. We can hypothesize that the reduced uptake of bile acids in

the terminal ileum occurring during cholestasis or the reduced bile acid secretion occurring in PSC, could lead to a secondary feedback down-regulation of colonic FXR; this would make more sense in advanced disease but unfortunately no data about PSC stage or serum bilirubin levels amongst the UC-PSC group was available.

Recent animal experiments have shown that activation of FXR in the intestine protects the liver from cholestasis by reducing the hepatic pool of bile acids[182]. Failure to activate FXR or constitutive down-regulation of FXR could therefore exacerbate PSC and hepatic cholestasis by increasing bile acids levels within hepatocytes. In a recent study, 2,355 IBD patients (1,193 with UC) and 853 controls were genotyped with seven tagging SNPs and two functional SNPs for FXR. None of the SNPs was associated with the presence of IBD; however, no information or sub-analysis for patients with PSC-UC was provided[175]. Ongoing[183] and future GWAs in PSC will probably shed some light on the role of FXR and other nuclear receptors and their role for PSC development and progression. It is apparent that the cross talk between the liver and the colon in PSC-UC patients has yet to be explored and FXR may be just another piece of the puzzle.

CHAPTER 3

THE FEATURES OF MUCOSA-ASSOCIATED MICROBIOTA IN PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

INTRODUCTION

The human microbiome is defined as the collection of organisms and their genomes inhabiting different locations and habitats, and includes not only bacteria but also viruses, archaea and fungi[184]. It is now known that our microbiome is crucial to our health and well-being[185]. The number and variety of bacteria exponentially increase from the proximal to the distal gastrointestinal tract, with the colon harboring most of the gut microbiota[185]. The human colon alone contains around 10^{11}-10^{12} different bacterial species, with anaerobic bacteria contributing 99% of the total diversity[186]. It is estimated that there are 100 times more bacterial genes in our body than human genes.

Colonization with commensal organisms begins shortly after birth on exposure to vaginal microbiota, and some authors have even hypothesized that bacterial colonization may start *in utero*[185, 187]. During the first years of life the developing microbiome is very dynamic and sensitive to environmental incursions, being formed as infants are introduced to new flora through breastfeeding, introduction of solid food, etc. After the first 2 to 3 years of life the microbiome acquires an adult-like configuration and it becomes more resilient to change and more stable[188]. Several factors are known to influence the gut microbiome composition such as antimicrobials, sanitation, vaccination, diet, and disease[186].

Intestinal microflora is essential for host wellbeing by virtue of their participation in immune and metabolic functions. For example, our gut bacteria are responsible for degradation of complex polysaccharides with subsequent production of short-chain fatty acids, which contribute to salvage of nutrients and to the integrity of the epithelial layer. The flora also have a role in preventing colonization of harmful pathogenic species, and they can participate in the detoxification of various carcinogens such as heterocyclic aromatic amines and polycyclic aromatic hydrocarbons[189]. Importantly, our gut flora has a major role in the mucosal immune system development and maturation. Indeed, while our immune system impacts microbiome composition and activity, the microbiome itself also modulates both innate and adaptive immune responses at the mucosal interfaces[190]. The interaction of our microbiome with the immune system results in several processes such as the as the secretion of secretory IgA and the release of endogenous antimicrobial peptides, as well the differentiation of T helper cells, especially T_{REG} (T regulatory cells) and Th_{17} cells (T helper), among others[190][186]. Changes in the bacterial communities in the gut have been associated with several conditions, such as colorectal cancer, obesity, liver diseases and inflammatory

bowel diseases[184], although it is not clear whether these changes are cause or consequence of disease status.

The 4 predominant bacterial phyla in our gut are *Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria*, followed by archaea, viruses, and fungi[185]. Most the bacteria present in our gut are non-cultivable or slow-growing, and therefore, for many years our knowledge about the microbiota was hampered by the limitation of traditional microbiological techniques in defining its complexity and diversity. However, in the last years the field of microbiome has witnessed major advances due to the development of high-throughput next-generation sequencing (NGS). One of the most commonly used NGS methods is the sequencing of 16s rRNA gene. The 16s rRNA gene is an evolutionary conserved gene amongst all bacterial species. It has hypervariable (the most commonly used being the V1–V3, V4, and V4–V5 regions) and conserved regions. Polymerase chain reaction (PCR) primers targeting broadly conserved regions of the 16S gene are used to amplify the microbial species, distinguishing bacterial from human DNA, while the sequencing of the hypervariable regions can be used to distinguish among bacterial genus/species[191]. After sequencing, the analysis of the 16S data involves assigning the DNA sequences to a given species, using the reference data from available libraries (eg: Greengenes, RDP, SILVA). The sequencing of the 16s rRNA gene generates usually thousands of reads. However, it is incorrect to assume that each unique read represents a different species: mutations and sequencing errors can lead to slightly different amplicon sequences within a species. Because not all DNA sequences match 100% the genomic sequencing and therefore can be confidently assigned to a given species, a new term has become common in taxonomy—the *operational taxonomic unit* (OTU)—which is used to characterize a group of microbes that share a certain level of 16S rRNA sequence homology[191, 192]. Generally, a 97% identity cutoff is typically used as an approximation of genus/species-level resolution; that is, sequences that share more than 97% similarity are assigned to an operational taxonomic unit (OTU) that is then classified to known bacteria[193]. After assigning reads to a taxonomic classification, several pipelines for data analysis exist, allowing the ecological characterization of a microbial community.

Multiple clinical and experimental observations, support a central role for the gut microbiota in IBD pathogenesis[194]. Studies in patients have consistently shown that that IBD is

characterized by a decrease in species diversity, altered bacterial function and aberrant bacterial communities in the gut, with a decrease in beneficial taxa namely in *Bacteroides* and *Firmicutes* (and specifically in bacteria from the *Clostridium cluster XIVa and IV),* and an increase in *Proteobacteria*[195]. The descriptions of dysbiosis in first-degree relatives of IBD patients or in unaffected twins of IBD probands[196, 197], and the presence of circulating antimicrobial antibodies in the blood of patients many years before diagnosis[17, 198], further suggest a primary role for the gut microbiota in determining disease risk; however, whether IBD gut-associated dysbiosis has a primary role in disease initiation and expansion, or merely reflects chronic inflammation, has so far been difficult to prove in humans[194]. Interestingly, it has been demonstrated that IBD patients present altered BA metabolism (dysmetabolism): patients with IBD present lower fecal and serum secondary BA proportions in comparison to healthy controls, due to a dysbiosis with a decrease in bacteria bearing BA hydrolase activities[199].

PSC could also potentially be associated with a specific gut flora. Some data from basic and clinical studies support the hypothesis that the intestinal microbiota may have a role in disease pathogenesis[200, 201]. For example, animal models of surgically-induced small intestinal bacterial overgrowth develop abnormalities in the intra- and extra-hepatic bile ducts that resemble PSC by cholangiography and histology[200]. The improvement of liver enzymes following a trial of antibiotic therapy, and a recent case reporting laboratorial and histological abnormalities reversal following vancomycin treatment in a liver transplant PSC recurrence, also suggest a role for the gut flora in disease pathogenesis[72, 202, 203]. With the advent of culture-independent techniques, a better understanding of how the gut microbiome can affect and modulate the development of liver diseases has emerged. Indeed, microbiota alterations in cirrhosis are now well-documented[204]. Being a cholestatic liver disease with profound alterations in bile acid pool[205], and acknowledging the reciprocal relationship between gut flora and bile acid metabolism[80, 206-208], it is very likely that patients with PSC-IBD exhibit alterations in their gut microbiota.

RATIONAL AND AIMS

Rational: It is plausible that different microbial communities could be associated with PSC-IBD as compared to IBD, and that this altered mucosal microbiome could contribute to their specific phenotype.

Aim: To explore whether different microbiomes across different colonic locations could be associated with the special phenotype of PSC-IBD, including more right-sided inflammation.

Approach: Patients with IBD and with PSC-IBD ongoing colonic surveillance at Mount Sinai Hospital and Chicago University were recruited and consented for biopsy collection for microbiome study.

Contribution of the PhD Candidate

The PhD candidate wrote the protocol and applied for funding while she was at Mount Sinai in 2011. She developed the standard operating procedures for the protocol, recruited patients and samples. When she came back to Portugal in 2012 the protocol was integrated in the SHARE (Sinai-Helmsley Alliance for Research Excellence Network) research alliance and the recruitment of patients and samples continued up to 2015 which coincided with the candidate´s return to Mount Sinai. She was therefore able to go on with the protocol and finalize the study. She participated in the DNA extraction from the samples; supervised by Professor Hu she learned how to handle the data analysis, calculate diversity analysis and other metrics in microbiome analysis, and therefore participated in the bioinformatics analysis. In conjunction and with the supervision of Professor Jianzhong Hu and Professor Steven H. Itzkowitz she wrote and published the paper which was published in Alimentary Pharmacology and Therapeutics journal (**Impact Factor: 7.286**): ***"The features of mucosa-associated microbiota in primary sclerosing cholangitis.*** *Torres J, Bao X, Goel A, Colombel JF, Pekow J, Jabri B, Williams KM, Castillo A, Odin JA, Meckel K, Fasihuddin F, Peter I, Itzkowitz S, Hu J. Aliment Pharmacol Ther. 2016 Apr;43(7):790-801. doi: 10.1111/apt.13552"*

MATERIAL AND METHODS

Subjects and sampling

Between November 2011 and November 2014, patients with PSC, patients with IBD and healthy controls undergoing colonoscopy at the Mount Sinai Medical Center and at the University of Chicago, were prospectively recruited, within a collaborative multicenter program for integrated studies in IBD (Sinai-Helmsley Alliance for Research Excellence (SHARE) Network). Sample collection protocol was standardized across two institutions to avoid possible bias during sample collection and processing. The inclusion criteria were age greater than 18 years, confirmed diagnosis of PSC based on histology and/or abnormal cholangiogram (ERCP or MRCP), and confirmed diagnosis of IBD by conventional endoscopic and histological criteria[209]. Patients with a personal history of colectomy, a diagnosis of secondary sclerosing cholangitis, or concomitant infectious colitis at the time of colonoscopy were excluded. Patients with newly diagnosed PSC, who were scheduled for their initial colonoscopy to screen for IBD, and healthy controls undergoing screening colonoscopy, were also recruited.

Demographical and clinical information were recorded for every patient. During colonoscopy, disease severity was recorded [Mayo score for UC and SES-CD (simple endoscopic score for CD) for CD], and biopsies for colorectal neoplasia screening were obtained, according to current guidelines. On all subjects, biopsies were collected from the left colon (LC) for microbiota analysis, and in a subset of patients, biopsies were also collected from the terminal ileum (TI) and right (RC) for comparison of the microbiota features across different colonic locations. Biopsies were either snap frozen or stored in RNAlater® (Qiagen, Valencia CA) for subsequent analysis. All samples were analysed at Icahn School of Medicine at Mount Sinai, New York.

Tissue DNA extraction and 16S ribosomal RNA (rRNA) amplification

Tissue samples were transferred into bead tubes (MO-BIO, Carlsbad, CA) and homogenized using bead beating method. Homogenized tissue samples were further processed using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol (Qiagen, Valencia, CA). Total DNA concentration was determined with Qubit 2.0 Fluorometer (Life Technologies, Norwalk, CT). The phylogenetically informative V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using universal primer set 347F/803R[210]. The

primers were synthesized by IDT (Integrated DNA Technology, Coralville, IA). We used a dual-barcoding approach to label the 16S rRNA amplicons from each sample as described previously. Briefly, the 6-mer barcodes were attached on the 5' ends of both forward and reverse PCR primers so that 16S rRNA PCR amplicons from each sample contained a unique dual barcode combination. The 16S rRNA amplicons were further pooled with equal molarity and submitted for MiSeq 2x300 pair-end sequencing at high depth. The paired sequence readings were merged and filtered by size (>400bp) and quality score (>Q30) using CLC genomics workbench version 7. The processed readings were further split by dual barcode for each sample and assigned taxonomic classification using QIIME (quantitative insights into microbial ecology) pipeline 1.8.0[211]. Repeated measurements of the same sample were made to assess sequencing reproducibility. After processing, QIIME provided detailed operational taxa unit (OTU) tables containing the microbiota composition and abundance for each individual sample.

Metagenomic 16S rRNA data analysis

Microbial diversity is usually described in terms of within (alpha-diversity) and between (beta-diversity) samples diversity. Both terms were introduced by Whittaker in 1972 to describe ecological metrics in a community[212]. Broadly speaking the alpha-diversity can be defined as the species richness[212]. It can be characterized using the total number of species [(species richness: eg. Chao1 index, OTU total abundance, or Phylogenetic Diversity (PD)] or the relative abundances of the species (species evenness: eg. Shannon index), or indices that combine these two dimensions[212]. The beta-diversity metrics provides a measure of the degree to which samples differ from one another, or in another words, the overall bacterial composition of a bacterial community. It can reveal aspects of microbial ecology that are not apparent from looking at the composition of the individual samples. Beta-diversity measures can be quantitative (using sequence abundance, e.g., Bray-Curtis or weighted UniFrac) or qualitative (considering only presence-absence of sequences, e.g., binary Jaccard or unweighted UniFrac) [213]. Second, they can be phylogeny based (the UniFrac metrics) or not (Bray-Curtis, etc)[213]. Usually beta-diversity measures are visualized using ordination techniques, in non-metric multiple dimensional scaling (nMDS) plots or in principal component analysis (PCA) or principle coordinates analysis (PCoA) graphs, that allow to reduce the dimensionality of the microbiome data sets so that a summary of the data can be visualized in two or three-dimensional plots[213].

In this study, to characterize the gut microbiota, firstly the overall microbiota dissimilarities among all samples (beta-diversity), were accessed using the Bray-Curtis distance matrices and visualized by non-metric multiple dimensional scaling (nMDS) plots. The PerMANOVA test[214], with the maximum number of permutations = 999, was performed using the [Adonis] function of the R package vegan 2.0-5[215, 216] to test the significance of the overall microbiota differences between groups by PSC and IBD status. Secondly, the diversity of the microbial community within each sample (alpha-diversity), was measured using the Shannon Index as a metric to represent the species diversity[217]. Next, significant differential taxa features at the family and genus levels were selected using random forest algorithm, a supervised machine learning approach, using R *package* rfPermute and confirmed by Boruta feature selection (R *package* Boruta)[218, 219]. Only features that were consistent in both analyses were selected. The significance of the selected taxa was further tested by t-test. In addition, at the OTU level, we performed the log likelihood ratio test (QIIME command group_significance.py using g-test statistics) to further identify significant differential OTUs between PSC and the healthy controls using LC samples only. The resulted *p*-values were adjusted by the FDR (false-discovery rates) methods. We also compared the PSC vs. IBD-only at LC, RC and TI locations using the differential OTUs selected from LC samples.

Blautia-specific long 16S rRNA sequencing

Long 16S rRNA reads can further improve the taxa OTU inference[220, 221]. Therefore, we designed a 16-base-barcoded 404F/1263R primer pairs specifically for the *Blautia* genus based on 16S rRNA reference sequence of the *Blautia* genus (**Supplementary Table 1**). The ~860 bp-sized PCR amplicons were pooled for sequencing on the PacBio RS II. Sequencing data from PacBio was processed using the manufacturer provided program *smrtanalysis* v.2.1.1 (https://github.com/PacificBiosciences/SMRT-Analysis/). Circular consensus sequencing (CCS) reads were then filtered by size (>800bp) and the quality score (>Q30) using CLC genomics workbench version 7. After split by barcode for each sample, all filtered reads were processed using QIIME pipeline 1.8.0[222]. The generated OTUs were filtered to only keep OTUs assigned to the *Blautia* genus and with more than 100 counts of reads. We performed the G-test of independence[222] to determine whether *Blautia* OTU presence/absence is associated with PSC status at LC samples only, in which we combined healthy control and non-PSC IBD together as non-PSC group to compare with PSC.

Representative sequences from significantly differential *Blautia* OTUs were further aligned with the *Blautia* reference sequences to construct the maximum likelihood phylogenetic tree using UPGMA (unweighted pair group method with arithmetic mean) method and performed a pair-wise sequence alignment comparison

RESULTS

Study population

Between November 2011 and November 2014, 46 patients were enrolled at both centres: 20 with PSC (19 of which had concomitant IBD), 16 with IBD and 10 healthy controls. There were 27 males (61%) and the median age for the whole population was 47 years (IQR 33.5, 58). The mean age of each group was as following: 43.8 years for healthy controls, 50.3 for the IBD group, and 45.3 for the PSC group. No patient was on antibiotics at the time of colonoscopy.

Samples from two subjects (one IBD patient and one healthy control) were eliminated from further analysis due to over-contamination (>90% relative abundance) with *Escherichia coli*). Therefore, 44 patients were included in the final analysis: 20 patients with PSC (19 with PSC-IBD and one with PSC-only), 15 patients with IBD and 9 healthy controls (**Table 3**).

	PSC (n=20)	**IBD (n=15)**
Male (n, %)	16 (80%)	9 (60%)
Age (y) Median, (IQR)	47 (33.5, 59.3)	48 (34.5, 59.5)
Smoking status Never Ever Unknown	 13 (65%) 6 (30%) 1 (5%)	 10 (67%) 5 (33%)
Type of IBD UC/IBDU	 13 (65%)	13 (87%) 2 (13%)

CD No IBD	6 (30%) 1 (5%)	
Extent of IBD	**UC/IBDU** Extensive colitis – 100%	**UC** Extensive colitis- 12 (92%) Left colitis – 1 (8%)
	CD Colonic disease: 3 (50%) Ileocolonic disease:3(50%)	**CD** Colonic disease: 2(100%)
PSC duration; median years (IQR)	4 (2, 12.3)	-
IBD duration, median years, (IQR)	9 (4.8, 18.9)	9 (4.75, 22)
PSC Mayo score*, median, (IQR)	0.03(-0.63, 0.42)	-
Endoscopic score of inflammation¶		
Normal/quiescent	11	11
Mild	5	3
Moderate	4	1
Medications for IBD		
Unknown/No medications	5	-
5-ASA	8	10
Thiopurines	5	8
Anti-TNF	5	3

Table 3 - Clinical characteristics of PSC and IBD (the patient presenting PSC-only was included in the PSC group together with PSC-IBD). *The PSC Mayo score is a model that estimates patient survival in PSC including reproducible variables (age, bilirubin, albumin, aspartate aminotransferase, and history of variceal bleeding)[223]; it could not be calculated in two patients due to lack of information. ¶ For purposes of simplicity the endoscopic score for CD-SES was replaced in this table

by the subjective impression of the physician performing the colonoscopy into mild or moderate inflammation (the median CD-SES for the patients with CD in this study was 11.5, IQR 10-13). IQR: interquartile range; IBDU: inflammatory bowel disease unclassified; UC: ulcerative colitis; CD: Chron´s disease; SES-CD: simple endoscopic score for CD; 5-ASA: 5-aminosalycilates.

Biopsies from different colonic locations (TI, RC and LC) were available in 18 subjects (11 with PSC, 6 with IBD-only and 1 with PSC only), except for two cases, where the TI could not be intubated. The clinical characteristics of PSC and IBD patients are described in **Table 3**. The patient with PSC-only was analysed in the PSC group. One of the PSC-IBD patients had a history of liver transplant and another had a history of choledochojejunostomy for a dominant stricture. There was no history of colon surgery among any of the participants.

Samples

16S rRNA amplicons from 81 samples (44 LC, 18 RC, 16 TI and 3 technical repeats) were sequenced and a total of 9.3 million reads were generated after filtering by size and quality, as described in the methods section. On average, each sample contained ~110,000 reads. The repeated measurements showed Pearson correlation to be 99% at the genus level. We used the mean of the repeated measurements for further analysis.

The mucosa-associated microbiota is stable across different locations within each individual

Considering the distinct phenotype of more inflammation and a higher prevalence of neoplasia in the right colon observed in PSC-IBD patients, we specifically assessed if there were any differences in the spatial distribution of the mucosa-associated microbiota between the RC, LC and TI. Therefore, we compared the microbiota composition at three biopsy locations in the 18 subjects, from whom multiple locations were available. Our results (**Figure 13 A)** showed that although a few samples from the same subjects showed substantial variations, in general, the overall microbiota was consistent across all three sampling locations. The distance across locations within the same subject was significantly smaller than the distance between samples from the same location but from different subjects (mean=0.18 and 0.45, SD=0.13 and 0.17, respectively, p-value<0.05). No significant

differences in the species richness (measured by the Shannon index) were observed between PSC and IBD-only patients in all three locations (**Figure 13 B)**.

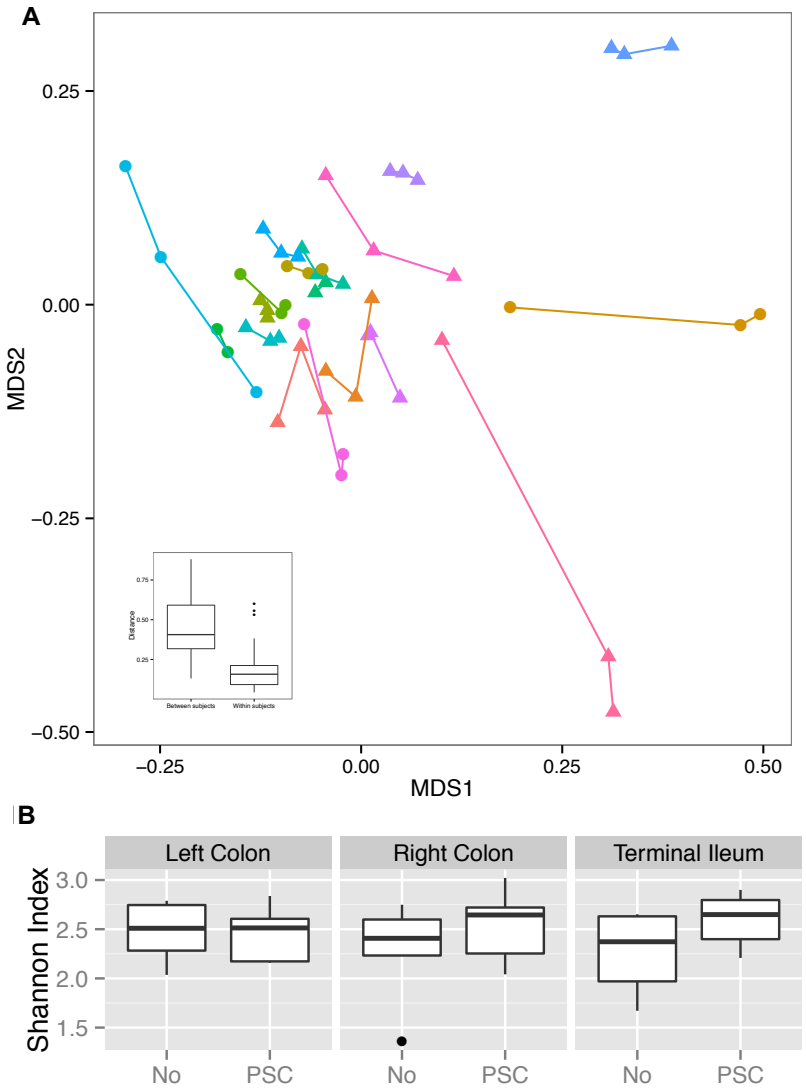


Figure 13 – Microbiota profiles across multiple biopsy locations. (A) The overall microbiota dissimilarities between samples are compared. Dissimilarities were measured by distance metrics (Bray-Curtis) and visualized using non-parametric multidimensional scaling plot. The closer a dot is to the other, the closer, the less dissimilar those samples are in relation to the others. Each color corresponds to a single subject and the lines are linking the multiple sampling locations from the same subject. Insert at lower left corner shows the mean and variance of the distance between (beta-diversity) and within (alpha-diversity) the samples from the same or different subjects. Triangles represent PSC patients; Circles represent non-PSC, IBD patients. It can be concluded that there are no significant differences between and within the samples from the same subject in different

locations. (B) The boxplots show the alpha-diversity calculated using the Shannon index, in the three different locations between patients with and without PSC. As it can be seen there are no significant difference across multiple locations.

PSC associated left colon mucosa microbiota features

Observing that there were no overall differences in the microbiota diversity between TI, RC and LC, we next analyzed the microbiota composition only in the LC of all subjects. The overall microbiota dissimilarities among all 44 LC samples (20 PSC, 15 IBD patients and 9 healthy controls) grouped by PSC and IBD status were accessed using the Bray-Curtis distance matrices (**Figure 14 A).**

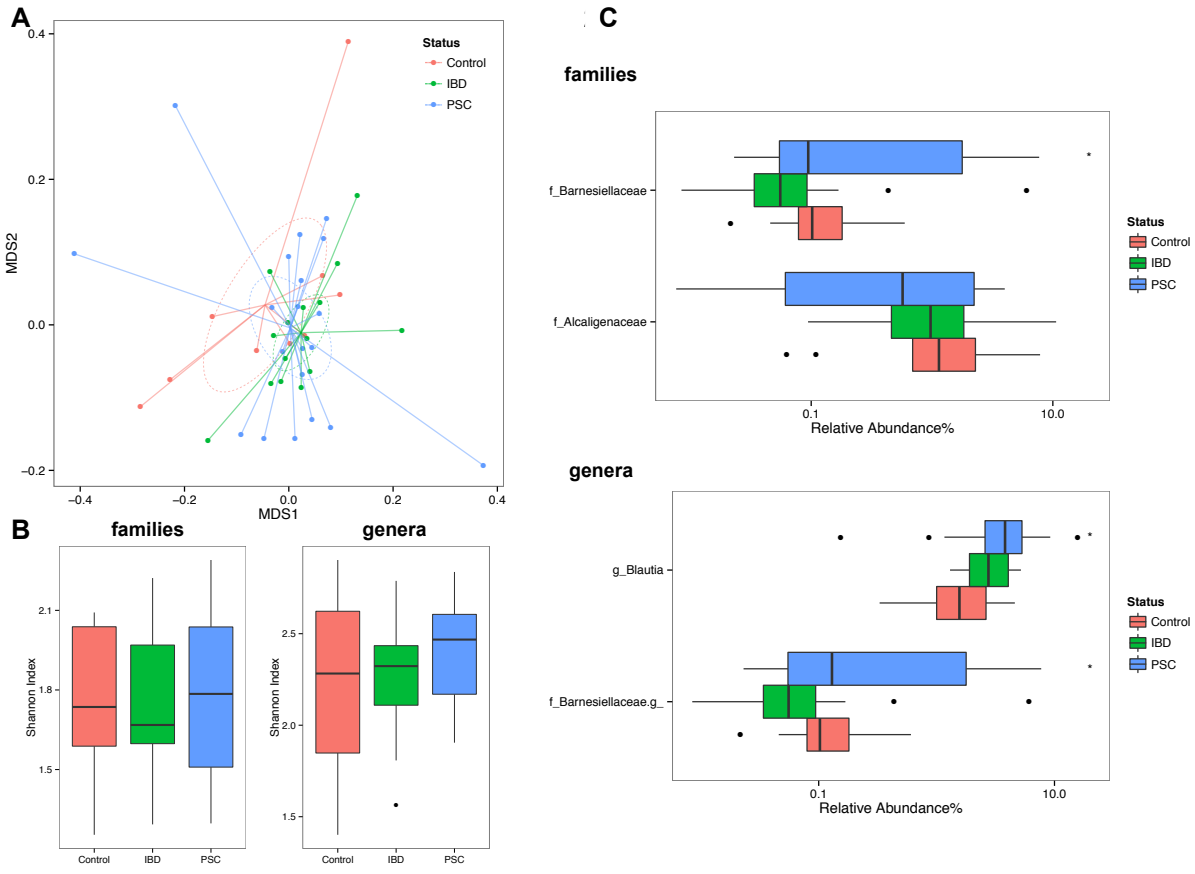


Figure 14 – Overall microbiota dissimilarities between samples grouped by PSC and IBD status, only in the samples from the left colon, representing all individuals in the study. Dissimilarities were measured by distance metrics (Bray-Curtis) and visualized using non-parametric multidimensional scaling plot. Although a separation in the different clusters can be seen, no significant differences were found between groups by the PerMANOVA test, using 999 permutations. (B) The boxplots showed the mean and variance of the richness of the microbial

community (alpha-diversity) calculated using the Shannon Index, between different disease status. No significant differences were seen neither at the family or genus level. (C) Taxonomic groups selected by random forest algorithm to be differentially expressed among groups are represented and the log of their relative abundance is plotted at the family (top panel) and genus (bottom panel) level. In the top panel, the bacterial family *Barnesiellaceae* is significantly enriched in PSC as compared to healthy controls. In the bottom panel it can be seen that the genus *Blautia* and an unidentified genus from the *Barnesiellaceae* is also significantly enriched in PSC as compared to healthy controls. The asterisk indicates the p-value <0.05 between PSC and healthy controls. In green are represented the samples from non-PSC IBD patients; in blue the samples from PSC patients and in pink the samples from healthy controls.

Although we noticed a separation between the healthy control and the IBD or PSC-IBD, the PerMANOVA test did not find a significant difference in the global LC microbiota profile by disease status. We also did not observe a significant difference among controls, IBD and PSC samples in species richness using Shannon Index (**Figure 14 B**). At the taxa level, two families including *Barnesiellaceae* and *Alcaligenaceae,* as well as two genera including *Blautia* and an unidentified genus from *Barnesiellaceae* family were selected by random forest algorithm using R package rfPermute and confirmed by Boruta feature selection (R package Boruta) (**Figure 14 C**). Among those selections, we observed significant enrichment of *Barnesiellaceae* family and its further assigned unidentified genus (mean abundance=1.3% in PSC samples, 0.48% in IBD and 0.16% in healthy controls; *p-values*=0.44, 0.025, respectively by t-test) and *Blautia* (mean= 4.5% in PSC samples, 2.9% in IBD and 2.1% in healthy controls*; p-values*=0.22 and 0.02, respectively by t-test) in PSC samples compared to healthy controls. To test whether or not the PSC-associated taxa features found in LC were consistent in the RC and TI, we further compared the abundance of *Barnesiellaceae* family and *Blautia* genus at three locations in the available samples from these locations. We found that not only did the enrichment of both taxa in PSC patients occur in all locations, but also that the abundance of those taxa was consistent across multiple locations within the same individual (**Figure 15**).

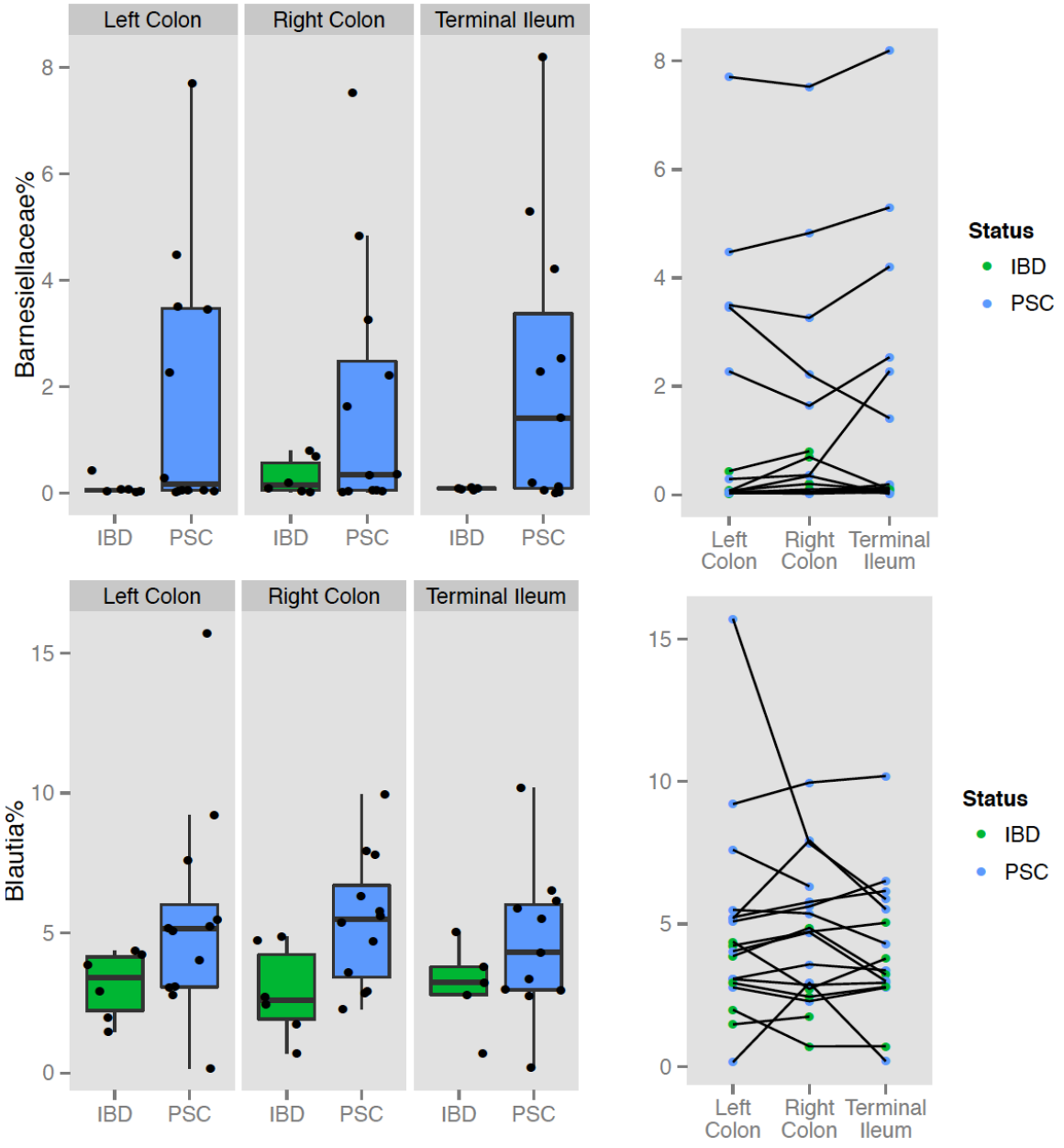


Figure 15 - The relative abundance of the *Barnesiellaceae* family and the *Blautia* genus in PSC and non-PSC-IBD at multiple locations. The boxplots (left panel) show the mean and variance of the relative abundance in the left, right colon and terminal ileum between PSC and no-PSC IBD. In the right panel the dot plots show the relative abundance for each individual samples and the lines link the samples from the same subject. Green: non-PSC IBD; blue: PSC

Further analysis excluding the patients with PSC with an history of OLT and an history of choledochojejunostomy did not change results (data not shown).

We then assessed whether there were any differences in microbiota composition according to PSC severity score. Based on the ranked PSC Mayo score, the PSC patients were assigned to low risk (Mayo score <0), intermediate risk (Mayo score from 0-2) and high-risk score (Mayo score>2). There were no patients with severe disease; however, among the low and intermediate risk, groups had a similar global microbiota composition (**Figure 16 A**) and taxa richness (**Figure 16 B**). At the taxa level, the level of *Blautia* was not different between the two different Mayo score risk groups. The low risk group showed higher median level of *Barnesiellaceae* family compared to the intermediate risk group (**Figure 16 C**), but this did not reach statistical significance.

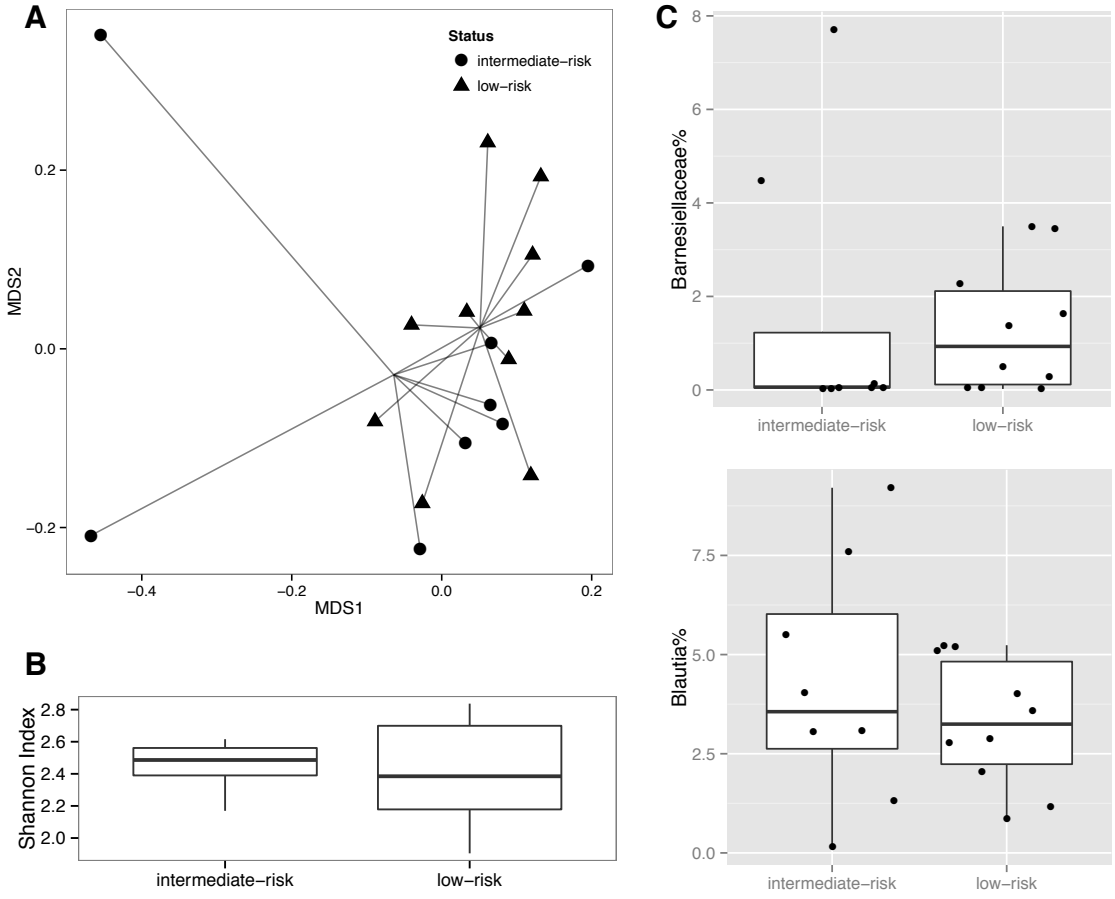


Figure 16 - Microbiome profiles and PSC disease severity. (A) The overall microbiome dissimilarities (beta-diversity) between samples with low-risk severity and intermediate risk severity as assessed by the PSC Mayo score are compared. As it can be seen there are no significant differences in the overall microbiome composition in patients with low or intermediate risk for liver disease progression. (B) The boxplots show the mean and variance of the richness of the microbial community within each sample (alpha-diversity) showing no significant difference according to

disease severity. (C) The boxplots show the mean and variance of the relative abundance of *Blautia* and *Barnesiellaceace* in patients with low-risk and intermediate risk PSC.

Differential OTUs by PSC status

We performed *de novo* OTU picking using QIIME pipeline. Based on the 97% similarity of the 16S rRNA sequencing reads, all sequencing reads were clustered into individual OTUs. After removing rare OTUs (relative abundance < 0.1% in all samples), we compared 2439 OTUs and selected 80 and 15 OTUs significantly ($p<0.05$ by parametric t-test, not adjusted) different between PSC, healthy control or IBD (**Supplementary Figure 1 and Supplementary Table 2**).

We found that when compared with healthy controls, most of the PSC-associated shifts in the bacterial composition were observed in the *Clostridiales* and *Bacteroidetes* orders, with 86% in the former order. In agreement with our above findings, several PSC-enriched OTUs belonged to the *Blautia* genus and the *Barnesiellaceae* family. When compared to IBD, many OTUs were from the *Blautia* genus. Similar enrichments and reductions at the PSC-associated OTUs selected from LC samples (**Supplementary Figure 2**) were observed in RC and TI locations.

Differential OTUs at Blautia genus between PSC and non-PSC by long-read 16S rRNA sequencing

Our results showed that both *Barnesiellaceae* and *Blautia* genus were enriched in PSC patients. But unlike *Barnesiellaceae*, *Blautia* comprised >2% of the entire microbiota regardless of the disease status, so it is plausible to specifically enrich this genus and further use the long 16S rRNA reads to perform additional taxa OTU inference at *Blautia* genus in both patient and control samples. After processing, 2967 OTUs were assigned to *Blautia* genus. Among those, 135 OTUs had reads of more than 100 counts. The G-test of independence further selected 7 out of 135 OTUs significantly different between PSC and non-PSC (unadjusted p-values<0.05). We aligned those *Blautia* OTUs with *Blautia* reference sequences and constructed the maximum likelihood phylogenetic tree using UPGMA method (**Supplementary Figure 3**).

We also performed the pair-wise sequence alignment comparison and showed the number of difference (upper) and the percent identity (lower) between pairs. (**Supplementary**

Figure 3 A). Our results showed that 4 OTUs (denovo17640, denovo28452, denovo13317, denovo6236) were enriched and 3 OTUs (denovo25871, denovo781, denovo18792) were reduced in PSC samples (**Supplementary Figure 3 B).** We also found that the OTUs denovo17640, denovo13317 and denovo28452 showed >99% percent identity to reference strains from species of *Blautia wexlerae*, *Ruminococcus obeum* and *Blautia faecis*, respectively (**Supplementary Figure 3 C**).

DISCUSSION

Herein we compared the mucosa-associated bacterial flora of patients with PSC to IBD alone and to normal subjects. Because fecal microbiota does not necessarily reflect the mucosa-associated microbiota[39, 224], we chose to examine the mucosa-associated microbiota, rather than stool, to gain more insight into the unique phenotype of PSC-IBD patients who tend to develop more right-sided inflammation, and more often proximal neoplasia as compared to IBD alone patients. We thought there might be some differences in the microbiota composition throughout different locations of the ileo-colon in this specific clinical context, given the phenotypic associations with disease location for PSC[64]. However, and in line with previous observations in healthy subjects[225], no significant site-specific differences in the microbial composition were found throughout the colon. Although this analysis may have been limited by sample size, it is possible that other mechanisms may be operating and interacting differently with the microbiota in the right as opposed to the left colon[226] in patients with PSC and IBD. Rossen et al[227] in a prior study, also described similar hierarchical clustering between samples from the TI and RC from the same subject from between PSC, IBD and healthy controls. However, no comparisons with the mucosa-associated from the left colon were available. The same authors described a lower diversity and abundance of uncultured *Clostridiales II* at the genus level compared to UC and healthy controls. However, this study, was limited by a smaller sample size and, by the use of a probe-based approach, the HITChip[227, 228] that only allows to detect phylotypes present in the chip at the genus-like level. Our study, using a longer read 16S sequencing, allowed us to provide deeper taxonomic analysis that could inform on specific microbiome shifts associated with PSC. Another recently published study[229], assessing differences in microbial composition PSC-UC and UC patients, did not report differences in overall microbial

diversity between PSC-IBD and UC, however different geographical provenience of samples was the main driver for microbial composition in this study. We observed a trend in the compositional dissimilarity of the overall microbiota between healthy controls, and PSC-IBD, but this did not reach statistical significance. This could be due to the modest sample size, underrepresentation of healthy controls, and disease remission for most of the IBD patients, since shifts in the microbiota can vary according to disease activity[230]. Using bacterial 16S rRNA next-generation sequencing, we found, across all colonic locations, a consistent PSC-enrichment in *Blautia and Barnesiellaceae* genera and shifts under the *Clostridiales*, and less frequently under the *Bacteroidales* order in comparison with healthy controls. Deeper taxa analysis at the OTU level was consistent with these findings, also showing several enriched OTUs particularly from *Blautia* and *Barnesiellaceae* genera. Specifically, around 86% of the relative changes in the microbiota at the OTU level occurred within the *Clostridiales* order. This is interesting, as shifts in these taxa have been observed both in IBD and in cirrhosis[206, 231]. *Bacteroidetes* species play an important role in protein metabolism[232] as well in bile acid deconjugation[233]. The *Clostridiales* order encompasses bacteria from *Lachnospiraceae* family, *Ruminococcaceae* family *and Blautia* genus, which can perform 7α-dehydroxylation[208], an important step in converting primary to secondary bile acids in the intestine. Furthermore, it has been demonstrated that certain *Clostridum spp.* can affect number, function and differentiation of colonic Treg cells, therefore playing a crucial role in colonic homeostasis[234]. In advanced cirrhosis, a shift in the gut flora towards the enrichment of *Enterobacteriaceae* and the reduction of *Clostridiales* and *Bacteroidetes* in parallel with a reduced level of fecal secondary bile acids has been described[206]. Conversely, Islam and colleagues showed that feeding animal models with bile acids leads to an enrichment of colonic bile acids, which in turns results in the expansion of the *Firmicutes* phylum, specifically within the *Clostridia* class, with *Blautia spp* expanding significantly[235]. Being a cholestatic liver disease, characterized by scarring of the bile ducts, PSC is expected to lead to a reduction of the flow of bile acids from the liver to the intestine, at least in the more advanced stages of the disease. However, there also are data suggesting that during obstructive cholestasis, the apical sodium dependent bile acid transporter ASBT is down-regulated[181, 236], as a feedback anti-cholestatic mechanism. This could hypothetically lead to a relative increase of BA in the proximal colon, which could in turn lead to an enrichment of bacterial species involved in bile acid handling[208, 236]. Since in our study we did not have patient´s bile acid profiles we couldn´t test for this hypothesis.

A limitation of our study is its relatively small sample size, so we were unable and unpowered to make any associations between disease severity and microbial composition, or to consider any impact of medication, diet, or disease course. Despite this limitation, and compared with previous studies, we were able to compare microbiota composition from multiple sites[229], had representation of PSC-IBD patients, IBD and normal controls[229], and a reasonable sample size[227], that allowed to perform deeper taxonomic analyses[227]. We observed that the mucosa-associated microbiota was consistent among all locations, and that the *Blautia* and *Barnesiellaceae* enrichment was consistently found not only in the left colon samples but also in other locations. Consistent with findings at the family and genus level, deeper OTU level analyses also found enrichment of *Blautia* and *Barnesiellaceae* OTUs in PSC. Therefore, in that sense we validated our findings. The cross-sectional nature of the study does not allow us to conclusively determine a causal link between the abundance of these species and its role in PSC. It is possible that the shifts in the microbiota features we observed in PSC-IBD are a consequence, rather than the cause, of the interaction between cholestasis and colonic inflammation. Furthermore, in the absence of a non-PSC liver disease control group, it is difficult to appreciate if the microbiota shifts we observed are specific to PSC or belong to a broader dysbiosis observed in chronic liver diseases[204, 206]. Whether these changes contribute to the special phenotype observed in PSC-IBD patients can only be speculated upon at this stage, and merits further investigation. Future studies investigating the role of microbiota in PSC should aim in collecting larger samples sizes, that could allow adjustment for clinical and analytical variables that could influence microbial composition in PSC such disease duration and stage, medication intake, diet, geographical location of patients, impact of liver transplant and or biliary surgery, as well as serum and fecal bile acid pool.

CHAPTER 4

THE GUT MICROBIOTA, BILE ACIDS AND THEIR CORRELATION IN PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

INTRODUCTION

Bile acids (BA) are produced in the liver, from cholesterol, conjugated with glycine or taurine, and thereafter secreted into the small intestine following a meal[204]. Normally, most of the BA secreted by the liver are efficiently reabsorbed in the terminal ileum, through an active process carried out by the apical sodium-dependent BA transporter (ASBT), leaving only approximately 5% of the total BA to reach the colonic lumen. In the colon, mostly on the right side, primary BA are transformed into secondary BA by bacterially-mediated deconjugation and dehydroxylation[161, 204]. Therefore, BA mediate communication between the liver and intestine[204]. BA are important not only for the absorption of dietary fats and vitamins, but they also are ligands for the nuclear receptor Farnesoid X receptor (FXR) and the G-protein – coupled receptor TGR5. The TGR5 is a G-protein couple receptor specific for bile acids, whose activation results in changes in intestinal motility, and has role in immunity. Work has shown that TGR5 activation lowers the pro-inflammatory cytokines interleukin-1α (IL-1α), IL-1β, IL-6 and tumour necrosis factor-α (TNF-α)[237].

Bile salts have antimicrobial properties[238] and have recently been shown, through FXR-activation, to regulate the expression of host genes whose products promote innate defense against luminal bacteria[165]. Bacteria have different tolerances to the actions of bile salts, and bacterial pathogenic mechanisms can be modified through interaction with bile salts [239]. Conversely, bile salt metabolism is a property of the gastrointestinal microflora; secondary BA, lithocholic (LCA) and deoxycholic (DCA) acid, are formed exclusively through microbial biotransformations in the large intestine[240]. It has also been shown that secondary BA have anti-inflammatory properties virtue of their activation of G protein-coupled specific membrane receptor TGR5[199]. However, some of the secondary bile salts generated by microorganisms can also be potentially toxic and/ or mutagenic or can lead to activation of other carcinogens in intestinal contents [239]. Exposure of cells of the GI tract to repeated high levels of BA is an important risk factor for gastrointestinal cancer, with the secondary BA, LCA and DCA acid being the most significant BA with respect to human colorectal cancer (CRC)[241]. This phenomenon has been widely demonstrated both in animal and in human studies. Patients with colonic adenomas and CRC present higher than normal concentrations of secondary and total BA in serum and feces [242]. BA can induce production of reactive oxygen and nitrogen species leading to DNA damage, mutation and genomic instability in colon cells [243]. The hydrophobic secondary BA, especially DCA acid, appears to be the most

strongly associated with CRC[241, 244]. Indeed, impaired absorption of BA in the small intestine has been related to colonic tumors. In one animal experiment, Kanamoto et al eliminated ASBT-mediated BA absorption by resecting the terminal ileum in rats. When these rats were then fed with DCA they had an increase in the influx of BA into the colon and a concomitant increase in the incidence of colonic tumors compared to controls [245]. Cholestatic liver diseases such as PSC are characterized by defective hepatic excretion of BA and their accumulation in serum and tissues. This excessive build-up results in the activation of anti-cholestatic responses in the kidneys, intestine and bile duct to provide alternative excretory routes and thus prevent hepatocellular accumulation of toxic components [246]. For example, it has been demonstrated, in both animal and human experiments, that during cholestasis, the ileal expression of ASBT is downregulated [236, 247]. These data suggest that intestinal BA absorption is reduced during obstructive cholestasis, which could lead to a relative increase of BA in the proximal colon, in turn producing heightened conversion of primary into secondary BA. Since concentrations of carcinogenic secondary BA are highest in the proximal colon, the finding of proximal cancers in PSC supports a role for these compounds[248].

RATIONAL AND AIMS

Rational: The net result of cholestasis and of a BA pool altered by the diverse bacteria in the inflamed colon are unknown but we can hypothesize that a specific alteration of the microbiome in PSC-IBD patients could promote the specific phenotype observed including colorectal carcinogenesis either directly or indirectly through BA transformation. Alternatively, changes in BA compositions could select more anti-inflammatory and/or pro-carcinogenic strains of bacteria in the gut.

Approach: Paired stool and fasting serum samples from patients with PSC-IBD and IBD alone will be collected and compared between groups. To assess whether specific BA-microbiome interactions exist, we will run correlation matrices in both groups and compare the differences.

Contribution of the PhD candidate

The PhD candidate developed the research idea and the protocol, under the supervision of Professor Marília Cravo and with the help of Professor Cecília Rodrigues. She applied and was granted with funding (GEDII investigation award 15.000 euros) that allowed her to complete the protocol. She contacted centers in Lisbon and developed a multicenter collaboration that allowed her to recruit the PSC-IBD patients. She collected samples and the clinical information. Samples were transferred to Mount Sinai Hospital in New York where she participated in the DNA extraction, library preparation and the bioinformatics analysis, supervised by Professor Hu. The extraction of serum and stool bile acids took place in Lisbon Pharmacy University under the supervision of Professor Cecília Rodrigues, and with the collaboration of PhD student Hugo Brito. The stool BA profile was examined in Paris in the lab of Professor Dominique Rainteau with the collaboration of Professor Jean-Frédéric Colombel. This collaborative work resulted in the presentation of this work in several national and international conferences and in its publication in the United European Journal (**Impact factor: 3.673**):" ***The gut microbiota, bile acids and their correlation in primary sclerosing cholangitis associated with inflammatory bowel disease.*** *J Torres, C Palmela, H Brito, X Bao, H Ruiqi, P Moura-Santos, J Pereira da Silva, A Oliveira, C Vieira, K Perez, SH Itzkowitz, JF Colombel, L Humbert, D Rainteau, M Cravo, CM Rodrigues, J Hu. United European Gastroenterology Journal, 6(1), 112-122, 2018 https://doi.org/10.1177/2050640617708953*".

In parallel with this project, and acknowledging the contribution of dietary patters to the gut microbiota, we conducted a separate study, assessing nutritional status and dietary intake between PSC-IBD patients and controls. This study was undertaken by Ana Paula Krieger, who under the supervision of Prof Marília Cravo and the PhD candidate used it for her Master thesis. Some of those results are presented here, and additionally the correlations between microbiome and dietary components, by study group were also explored.

METHODS

Subjects and samples

Between October 2014 and July 2015, 15 patients with PSC-IBD and 15 patients with IBD were prospectively recruited. The inclusion criteria were age greater than 18 years old, confirmed diagnosis of PSC based on histology and/or abnormal cholangiogram (ERCP or

MRCP), a confirmed diagnosis of IBD by conventional endoscopic and histological criteria, and the presence of extensive colitis. Patients with a personal history of colectomy, a diagnosis of secondary sclerosing cholangitis or a history of OLT were excluded. All patients provided clinical and demographic information, and completed a semi-quantitative food frequency questionnaire (FFQ) validated for the Portuguese population[249]. Nutritional assessment was also conducted by a dedicated nutritionist, and included standard assessments (weight, height) as well as bioimpedance measurement. The conversion of food into nutrients was carried out by the Institute of Public Health of the University of Porto, based on the Food Processor Plus (ESHA Research, Salem, Oregon) software, with nutritional information from the Food Composition Tables of the Department of Agriculture of the United States of America, adapted to typical Portuguese foods.

Clinical activity was scored according to the Mayo score for ulcerative colitis[250], and the Harvey-Bradshaw index for Crohn´s disease[251]. Endoscopic activity was scored according to the Mayo endoscopic score for UC[250] and the Simple Endoscopic Score for Crohn's Disease (SES-CD)[252]. All study participants collected a stool sample for BA analysis and microbiota analysis. All PSC patients on ursodeoxycholic acid (UDCA) therapy were required to stop it two weeks before specimen collection. A minimum interval of 3 months was required between antibiotic intake or bowel preparation (for colonoscopy) and sample collection. During colonoscopy disease severity was recorded, and biopsies for colorectal neoplasia screening were obtained, according to current guidelines.

Serum bile acid profiles

A fasting serum sample was obtained from each patient**.** Individual amidated bile acids in serum (1 mL) were determined by high-performance liquid chromatography (HPLC)[253], after solid-phase extraction using Sep-Pak C18 cartridges (Waters Corp., Milford, Massachusetts, USA)[254]. Identification of BA was made based on gas chromatography retention index relative to a homologous series of n-alkanes, and the chromatograms compared with authentic standards. Quantitation was achieved by comparing the peak height response of the individual BA with the peak height response obtained from the internal standard. Only the conjugates fraction of BA was measured in serum.

Stool Bile acid profiles

A morning stool sample was obtained and dried to obtain a lyophilized extract. To lyophilized fecal samples were weighing 1 g, 80% methanol was added. All samples were sonicated for 30 minutes, refluxed for two hours, and then cooled and filtered[255]. The residue was re-suspended in chloroform/methanol (1:1, v/v), refluxed for one hour, and filtered. The combined extracts were taken to dryness, and re-suspended in 10ml MeOH. An aliquot of 1ml was added with 2µl of 1mg/ml nordeoxycholic acid, and was diluted in 10ml deionized water and deposed on a 300mg HLB Oasis column, washed with 10 volumes deionized, 1 volume cyclohexane, and the BA were then eluted with 5ml MeOH which were taken to dryness and resuspended in 250µl MeOH. Four microliters were injected on the Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS) as previously described[256]. Results are reported in nmol/g of dried stool for total BA and in proportion of the median after calibration of the method, with weighted mixtures and normalization relative to the internal standard[256]. The conjugate and non-conjugated species were quantified.

Bile acid analysis

BA were not normally distributed according to the Shapiro-Wilk test; therefore, their distributions were compared using non-parametric tests. The relative proportion of a given BA corresponds to its concentrations divided by the total of BA. BA results are presented as the median proportion. For example, the total primary stool BA is the sum of CA and CDCA and their respective glyco-, tauro-, and sulphoderivatives. Linear discriminant analysis (LDA) was conducted to illustrate the classification of disease groups (IBD only and PSC-IBD) using stool BA. LDA is a dimension reduction statistical technique that looks for a combination of features (continuous variables) that maximize the separation between classes. LDA was performed using MASS package in R software.

Stool DNA extraction

Approximately 200mg of stool were transferred into bead tubes (MO-BIO, Carlsbad, CA) and homogenized using bead beating method. Homogenized stool samples were further processed using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol (Qiagen, Valencia, CA). Total DNA concentration was determined with Qubit 2.0 Fluorometer (Life Technologies, Norwalk, CT).

16S ribosomal RNA (rRNA) sequencing

The phylogenetically informative V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using universal primer set 347F/803R[210]. The primers were synthesized by IDT (Integrated DNA Technology, Coralville, IA). We used a dual-barcoding approach to label the 16S rRNA amplicons from each sample as described previously[257]. The 16S rRNA amplicons were further pooled with equal molarity and submitted for MiSeq 2x300 pair-end sequencing at high depth. The paired sequence readings were merged and filtered by size (>400bp) and quality score (>Q30) using PANDAseq (PAired-eND Assembler for DNA sequences)[258]. The processed readings were further split by dual barcodes for each sample and assigned taxonomic classification using QIIME (Quantitative Insights Into Microbial Ecology) pipeline 1.9.0[259]. Repeated measurements of the same sample were made to assess sequencing reproducibility. After processing, QIIME provided detailed operational taxa unit (OTU) tables containing the microbiota composition and abundance for each individual sample.

Data analysis

First, we measured the diversity of the overall microbiota communities within or across each sample. The overall species richness within each patient group, so-called alpha-diversity, was measured using the Chao1 and Shannon Index on rarefied tables at 8000 sequences per sample[260]. Beta-diversity was measured using unweighted and weighted UniFrac distance matrices on the rarefied tables. The permutational analysis of variance (PERMANOVA) test (number of permutations = 999), was performed using QIIME command compare_categories.py to test the overall microbiota differences between groups by PSC and IBD status[261]. Secondly, at the taxa level, the LDA Effect Size (LefSe) analysis was used with default parameters to select taxa features that were associated to PSC status[262]. LEfSe combines robust tests for measuring statistical significance (Kruskal-Wallis test) with quantitative tests for biological consistency (Wilcoxon-rank sum test). The differentially abundant and biologically relevant features (clades, genes, pathways, functional categories) are ranked by effect size after undergoing LDA[263]. Only features with LDA score >2.0 were kept. A Kruskal-Wallis test on the LefSe selected differential taxa at the genus level was performed and corresponding p-values were adjusted for multiple comparisons. Finally, the Kruskal-Wallis test was also performed at individual OTUs to select OTUs with significant

differential abundance with respect to the PSC-IBD status. All singleton OTUs were removed prior to all analysis.

Correlation networks

We calculated the correlations between microbiota taxa and dietary components using the results obtained from the food-frequency questionnaires, stratified by study group. Pearson correlation was used to assess the relationship between nutrients and microbiota composition. P-values were adjusted for false discovery rate.

We also calculated both Pearson's and Spearman's correlations between the most abundant (mean relative abundance >0.1%) 65 genera in the gut microbiota and the stool BA levels in PSC and non-PSC IBD. To reduce the bias in the correlation analysis due to non-normality, we removed the variables with more than 8 null value results, and removed the measurements beyond the 5% quantile of the distribution. We computed the raw probabilities. The p-values of the Pearson's correlation were calculated using corr.test function in R software with FDR adjustment for multiple comparisons. Spearman's correlations of the selected pairs with significant p-values in the Pearson correlations were also computed to check the consistency of the correlations.

RESULTS

Study population

Thirty patients with IBD, of whom 15 had concomitant PSC, were prospectively enrolled. All patients enrolled had pancolitis; 2 out of the 4 patients with CD also had ileal involvement. Two of the 15 PSC patients had concomitant liver cirrhosis (Child-Pugh A, 6 points). No patient had a prior history of abdominal or liver surgery. There were no significant differences in the overall daily intake of macro or micronutrients as assessed by the food-frequency questionnaire (data not shown). Patients with PSC-IBD presented, as expected, significantly higher levels of cholestasis markers, and were more frequently medicated with ursodeoxycholic acid. No further significantly different clinical variables were found, except for body mass index (BMI) that was significantly lower in PSC-IBD patients (**Table 4**). The median interval between stool collection and colonoscopy was 17.5

days (9-62). The additional demographic and clinical characteristics of PSC and IBD patients are described in **Table 4.**

	PSC-IBD (n=15)	IBD (n=15)	p value*
Male (n, %)	5 (33%)	10 (67%)	0.068
Age (y) (Median, IQR)	42(24)	45 (13)	0.62
Smoking status (n, %)			
Never	12 (80%)	12 (80%)	1.0
Ever	3 (20%)	3 (20%)	
Type of IBD (n, %)			
UC	11 (73%)	12 (80%)	0.6
CD	4 (27%)	3 (20%)	
PSC duration median years (IQR)	7.8 (11.7)	-	-
IBD duration median years, (IQR)	11.4 (5.26)	11.1 (15.7)	0.78
PSC Mayo score median, (min, max)	-0.57 (-1.6, 1.7)	-	-
ALP (UI/L) (median, IQR)	200 (166)	54 (28)	<0.001
GGT (UI/L) (median, IQR)	332 (414)	28 (20)	<0.001
CRP (mg/dL) (median, IQR)	0.2 (0.7)	1.1 (1.3)	0.061
Disease clinical activity (n, %)			
Remission-Mild	13(87%)	15(100%)	0.483
Moderate-Severe	2 (13%)	0	
Disease endoscopic activity (n, %)‡			
Remission-Mild	9 (64%)	13 (87%)	0.215
Moderate-Severe	5 (36%)	2(13%)	
Mean BMI (Kg/m^2)	24±4.5	30.1±6.4	0.005

Presence of colorectal dysplasia (n, %)	3/14(21%)	1/15 (6%)	0.2
Medications (n, %)			
5-ASA	12 (80%)	11 (73%)	1.0
Thiopurines	5 (33%)	8 (53%)	0.27
Anti-TNF	3 (20%)	3 (20%)	1.0
UDCA	10 (67%)	-	<0.001

Table 4 – Demographic and clinical characteristic of patients. *Variable distribution was compared using the t-Student´s test, the Mann-Whitney test or the χ^2 test, as appropriate. ALP: Alkaline phosphatase; GGT: Gammaglutamyl transpeptidase; CRP: C-Reactive Protein. IQR: interquartile range. BMI: body mass index. 5-ASA: 5-aminosalicilates. Anti-TNF: anti-Tumour necrosis factor. ‡: in the PSC-IBD group one patient refused colonoscopy.

Nutritional assessment and nutrient intake

As compared to IBD patients, PSC-IBD presented significantly lower weight and BMI. The remaining nutritional parameters were similar.

Measurements	PSC-IBD	IBD	P value
Weight (kg)	65,3 ±14,0	83,2 ±19,4	0,009
Height (m)	1,65 ±0,10	1,66 ±0,10	0,595
BMI (kg/m^2)	23,9 ±4,5	30,1 ±6,4	0,005
Fat mass (%)	27,9 ±10,2	30,4 ±9,5	0,436
Lean mass (%)	72,1 ±10,2	69,6 ±9,5	0,436
Total body water	56,0 ±10,0	51,7 ±6,8	0,116

Table 5 - Nutritional assessment parameters and bio-impedance results.

No significant differences were found in the daily intake of macro and micronutrients among groups (**Tables 6 and 7**).

Nutrient	PSC-IBD	IBD	P value
Energy (kcal)	2330 ±626	2154 ±708	0,367
Kcal/kg	37,6 ±13,2	26,8 ±9,0	0,019
Proteins (g)	90,9 ±26,2	85,9 ±29,5	0,744
Carbo-hydrates (g)	272,1 ±81,6	248,9 ±82,3	0,202
Complex carbohydrates (g)	79,1 ±30,7	87,6 ±58,9	0,870
Sugars (g)	116,0 ±46,0	101,9 ±29,8	0,367
Total fiber (g)	23,5 ±10,6	23,5 ±8,9	0,870
Total fat (g)	102,0 ±39,5	92,7 ±41,3	0,539
Total saturated fat (g)	29,0 ±10,9	25,3 ±10,8	0,512
Monounsaturated fat (g)	50,4 ±23,0	44,7 ±22,3	0,595
Polyunsaturated fat (g)	14,9 ±6,5	15,4 ±8,2	0,870
Cholesterol (mg)	301,9 ±110,3	319,5 ±139,8	0,595
Fatty acids n-3 (g)	1,40 ±0,42	1,42 ±0,63	0,902
Fatty acids n-6 (g)	11,97 ±5,82	12,37 ±7,06	0,902
Ratio n-6 / n-3	8,37 ±2,52	8,69 ±3,39	0,838

Table 6 – Daily ingestion of macronutrients between PSC-IBD and IBD alone. No significant differences were found between groups.

Nutrient	PSC-IBD	IBD	Valor de p
Vitamin A (µg)	1887 ±1135	1778 ±846	0,806
Vitamin D (µg)	3,10 ±0,70	3,54 ±2,04	0,683
Vitamin E (mg)	12,29 ±5,01	11,59 ±5,49	0,567
Vitamin K (µg)	8,85 ±7,67	12,58 ±10,41	0,285
Vitamin B12 (µg)	7,98 ±2,80	8,34 ±3,28	0,567
Vitamin C (mg)	120,1 ±79,4	120,5 ±83,8	0,902
Folate (µg)	286,9 ±120,5	265,6 ±81,3	0,512
Iron (mg)	14,91 ±4,60	14,05 ±4,96	0,436
Magnesium (mg)	307,8 ±106,8	301,2 ±107,2	0,713

Potassium (g)	3226 ±1036	3109 ±961	0,870
Iodine (µg)	49,8 ±40,9	35,4 ±28,6	0,305
Phosphor	1288 ±396	1198 ±406	0,367
Calcium (mg)	872,0 ±388,3	678,7 ±273,6	0,126

Table 7. Daily ingestion of micronutrients between PSC-IBD and IBD alone. No significant differences were found between groups.

Serum Bile acid

The total serum bile acid (µmol/L) pool was significantly expanded in PSC-IBD (p-value = 0.007, Mann-Whitney test) (**Table 8**). No significant differences were seen in the proportion of individual bile acids between groups. There was a positive correlation between PSC duration and total serum bile acids (ρ=0.66, p=0.009).

Bile acids	PSC-IBD	IBD	P value
Total bile acids (µmol/L)	18.5(30.6)	8.2 (7.0)	0.007
Total primary bile acids	80 (20)	70 (20)	0.20
Total secondary bile acids	7.3 (5.6)	9.5 (5.2)	0.84
Tauro/glyco-conjugates	4.7 (4.7)	3.8 (4.4)	0.53
CA	18.5 (79.2)	40.4 (63.9)	0.683
CDCA	42.0 (72.2)	38.4 (70.1)	0.325
DCA	6.1 (5.8)	5.8 (11.5)	0.486
LCA	1.5 (3.3)	2.5 (6.4)	0.174
UDCA	9.4 (41.2)	19.1 (30.4)	0.074

Table 8- Serum bile acids in PSC-IBD and IBD patients. Total Bile acids are expressed in (µmol/L). The other analytes are expressed as %median (interquartile range) of total BAs. Distributions were compared with non-parametric tests (Mann-Whitney).CA – cholic acid; CDCA – chenodeoxycholic acid; DCA – deoxycholic acid; LCA – lithocholic acid; UDCA – ursodeoxycholic acid

Stool bile acids profiles

The median total stool BAs were significantly reduced in PSC-IBD (167.2 µmol/L in PSC-IBD versus 282.4 µmol/L in IBD, p=0.021). Overall there were no significant differences in the proportions of each BA (**Table 9)** although the overall combination of stool allowed a good separation between PSC-IBD and IBD, as visualized in the linear discriminant analysis (**Figure 17**).

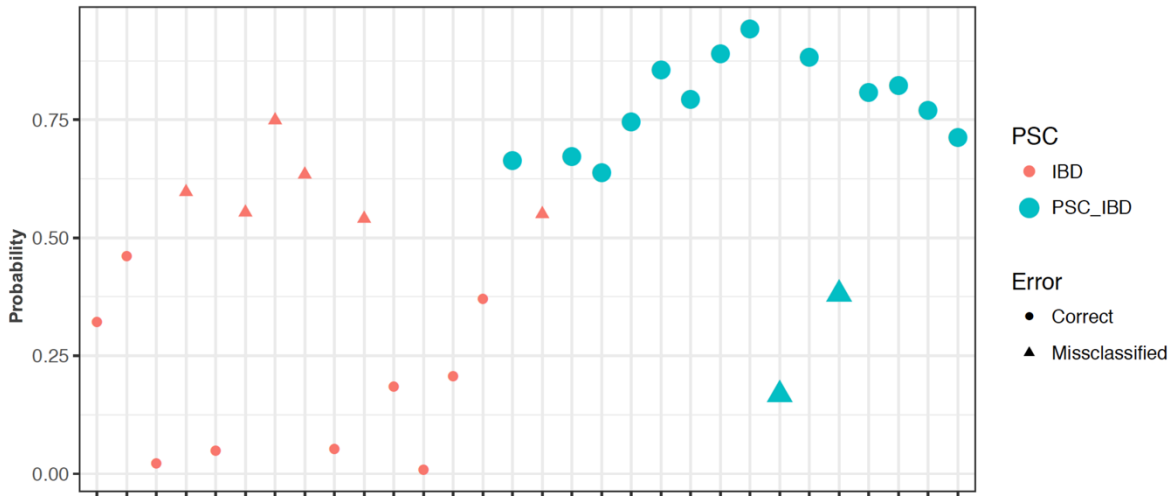


Figure 17 - Results of the linear discriminant analysis allowing to see the discrimination of PSC-IBD versus IBD alone, based on the combination of the main bile acids present in stool (CA, CDCA, LCA, DCA and UDCA). In the *x* axis each marker represents a sample from a patient. In the *y* axis is represented the probability of being correctly classified as PSC-IBD using the BA analytes. The green markers represent patients with PSC-IBD and in the pink markers patients with IBD. The circle represents patients that were correctly assigned to their disease group. The classification accuracy of the LDA was 73%, with a sensitivity and specificity of 86.7% and 60% respectively.

Using the main bile acids BA (CA, CDCA, LCA, DCA and UDCA), the classification accuracy of the LDA was 73%, with a sensitivity and specificity of 86.7% and 60% respectively (**Figure 17**). When we used all individual BA (taurine and glycine conjugates and sulphated BA), the accuracy of the LDA for classifying PSC-IBD versus IBD was 100%(**Supplementary Figure 4 A**). Additional LDA analysis was conducted using the top four most discriminatory stool BA (**Supplementary Figure 4 A)** and showed an accuracy of 70%.

PSC-IBD patients presented a higher proportion of conjugated BA, although this did not reach statistical significance. DCA, a secondary BA, was also elevated, albeit non-significantly, in PSC-IBD. The proportion of UDCA in stool was not different in the PSC patients who had been medicated with UDCA versus those who were not (1.075 nmol/g versus 1.35 nmol/g, respectively, p-value=0.7, Mann–Whitney Test) but like stated in material and methods section, all patients were asked to stop UDCA therapy for 2 weeks prior to stool collection. Likewise, the results for all stool BA comparisons did not change after excluding the 2 CD patients with ileal involvement (data not shown). There was a negative correlation between the concentration of secondary BA and endoscopic disease activity (ρ=-539, p=0,003); this was also observed when the analysis was stratified by patient group (data not shown).

Bile acids	PSC-IBD	IBD	P value
Primary BAs	9.5 (18)	4.2 (15.2)	0.29
Secondary BAs	89.4 (24.6)	91.2 (15.1)	0.57
CA	4.6 (6.45)	1.49 (4.77)	0.06
CDCA	4.73 (10.4)	2.72 (11.0)	1.0
DCA	52.5 (23.5)	43.6 (14.3)	0.55
LCA	34.1 (33.8)	46.2 (1.9)	0.14
UDCA	1.1 (1.9)	1.8 (3.9)	0.37
Tauro/Glyco conjugates	0.47 (0.67)	.34 (0.69)	0.98
Sulfated BAs	2.1 (3.1)	2.4 (16.3)	0.41
Conjugated BAs	4.5 (13.7)	2.7 (6.9)	0.23

Table 9 - Stool bile acids (BA) in PSC-IBD and IBD patients. BA are expressed as %median (interquartile range) of total BAs. Distributions were compared with non-parametric tests (Mann-Whitney). Due to a small amount of minor BA species in stool (muricholic acid, hycholic acid, hyodeoxycholic acid, or ursodeoxhycolic acid) which are considered by some authors as 'tertiary BA', the sum of primary and secondary bile acids is not 100%. CA – cholic acid; CDCA – chenodeoxycholic acid; LCA – litocholic acid; DCA – deoxycholic acid; UDCA – ursodeoxycholic acid.

Survey of gut microbiota

Using 16S rRNA sequencing, we surveyed the microbiome composition of 30 stool samples. The duplicate measurements showed Pearson correlation over 99% at genus level, confirming the reproducibility of the experimental approach. The overall microbiota dissimilarities among all samples grouped by PSC and IBD status were assessed using the UniFrac distance matrices (**Figure 18 A**). The overall qualitative microbial composition of patients with PSC-IBD was different as observed in the multidimensional scaling (MDS) plot (**Figure 18 A**: unweighted UniFrac, PERMANOVA: pseudo-F statistic: 2.99, p value=0.008). PSC-IBD presented lower alpha-diversity, albeit not significantly different (Chao1 899.3 for IBD vs 832.0 for PSC-IBD, p-value=0.36; Shannon index 5.7 for IBD vs 5.3 for PSC-IBD, p=0.23) (**Figure 18 B**). Patients with PSC and concomitant cirrhosis (n=2) presented significantly lower bacterial alpha-diversity (p=0.005) as compared to those with PSC without cirrhosis (data not shown). At the individual taxa level (**Figure 18 C**:), we found 5 genera differentially expressed in PSC-IBD vs IBD (logarithmic LDA score>2 by LEfSe analysis): *Ruminococcus*, and *Fusobacterium* were more abundant in PSC-IBD, while *Dorea, Veillonella, Lachnospira, Blautia*, and *Roseburia* were less abundant.

All of those genera were found to be significant ($p<0.05$) when their relative abundance was compared using a Kruskal-Wallis test (p values adjusted for multiple comparisons). No significant differences in the microbial overall composition (ß-diversity) were observed by PSC disease severity (as measured by the PSC Mayo score), BMI, UDCA use, IBD type, or IBD disease activity (data not shown).

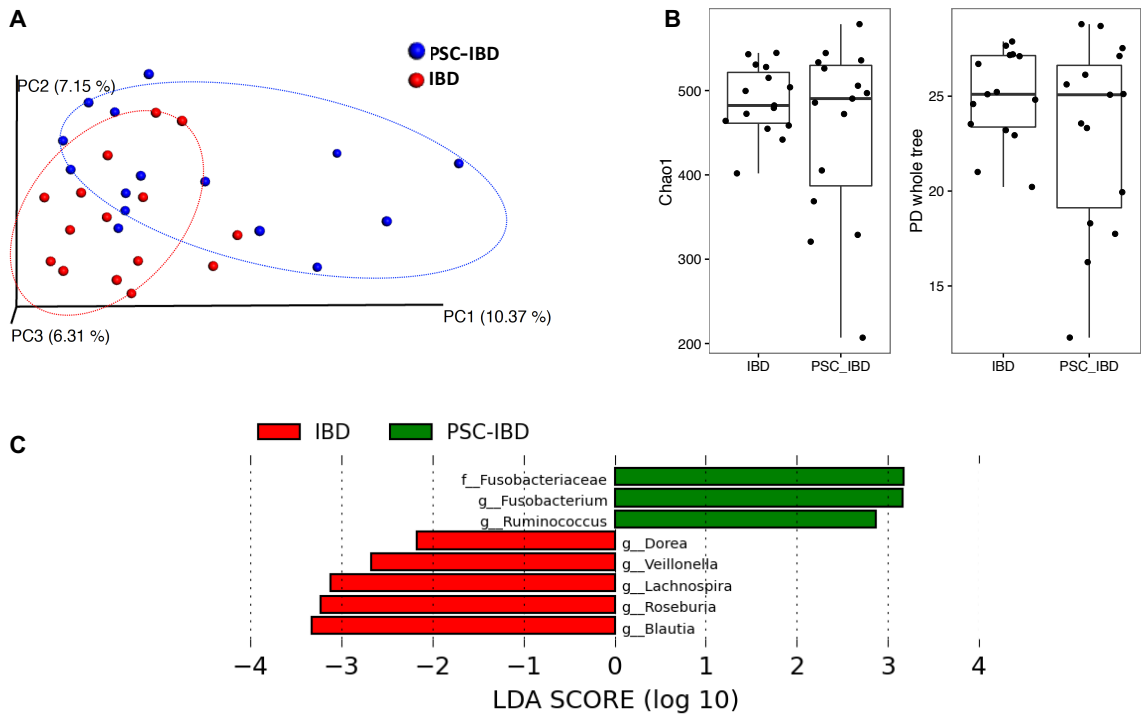


Figure 18 - Overall microbiota dissimilarities between samples grouped by PSC and IBD status. (A) Dissimilarities were measured using UniFrac unweighted distances and visualized using a multidimensional plot (MDS) plot. The blue circle represents patients with PSC-IBD, while the red circle is circling the samples from IBD patients. The PerMANOVA test (permutations= 999= showed that the distances between samples from both groups were significantly different (p= 0.008). (B) The boxplots show the mean and variance of the richness of the microbial community between different disease status (Chao1 in the left and and PD_whole tree index on the right); no significant differences are seen (p value: 0.36 and 0.23 respectively). (C) Top discriminative bacteria in PSC-IBD and IBD patients as determined by LEfSe analysis (LDA, linear discriminant analysis). In the right are represented the taxa increased in PSC-IBD, while in the left the taxa decreased in PSC-IBD, as compared to IBD.

Differential OTUs by PSC status

Based on 97% similarity of the 16S sequencing reads, the open-reference OTU picking using QIIME pipeline assigned all sequencing reads into individual OTUs. After removing singletons, we compared 3839 OTUs and selected 143 OTUs which were significantly ($p<0.05$ by Kruskal–Wallis test, not adjusted) differential and presented a >2 fold changes in the mean abundance between IBD and PSC-IBD (**Figure 19**).

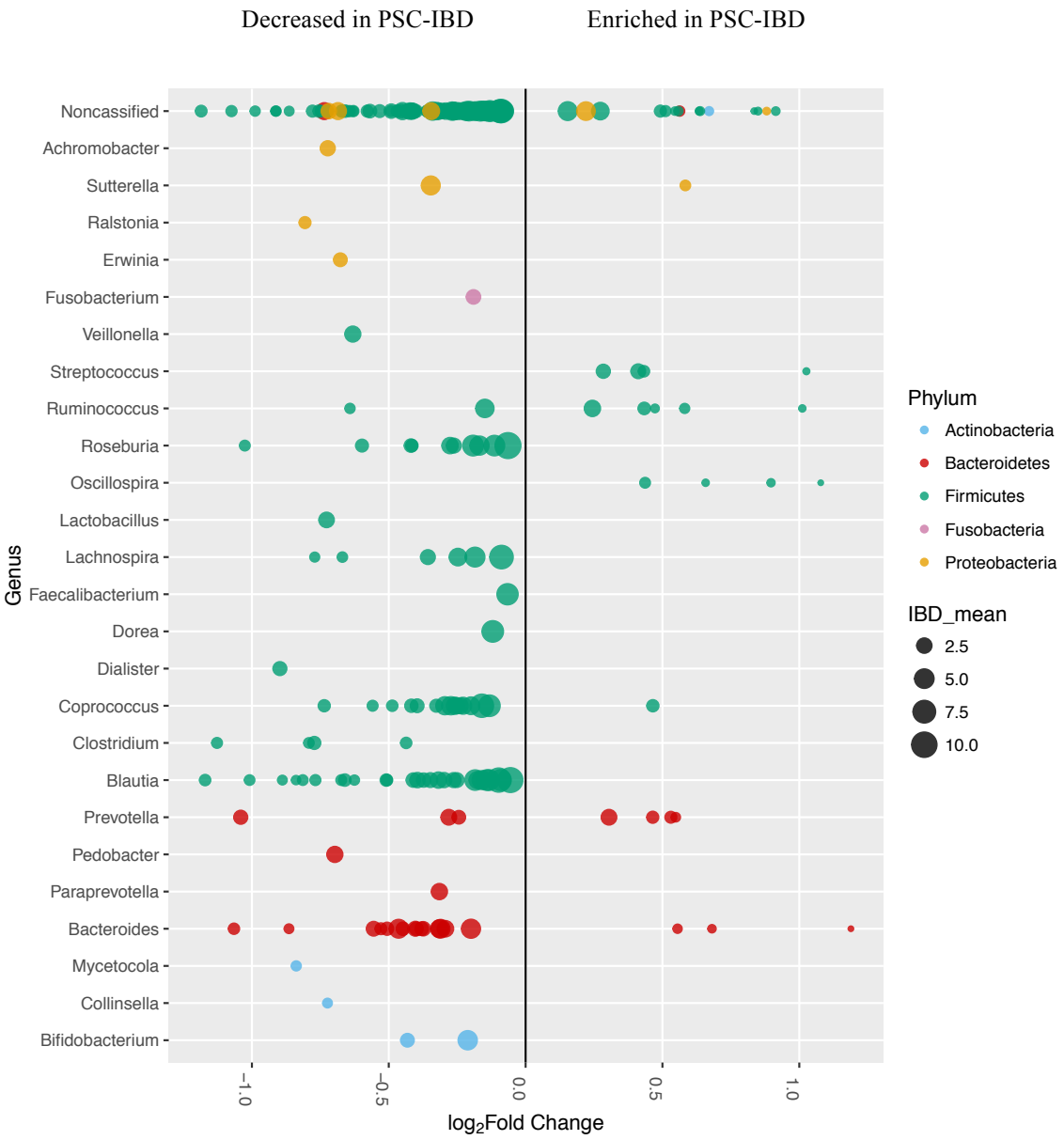


Figure 19 - Differential OTUs by PSC status. The dot plot shows the 225 OTUs with p-value <0.05 by Kruskal–Wallis test selected from 26 genera and coloured by their assigned phyla. The fold changes are calculated using the mean relative abundance of OTUs in IBD divided by the mean relative abundance of OTUs in PSC. The area of each dot was scaled to the mean relative abundance of represented OTU in IBD.

Compared to IBD only, the relative abundance of 32 OTUs were increased and 111 OTUs were decreased in PSC-IBD. At the phylum level, we found that most of the shifts associated with PSC occurred within the *Firmicutes* (73%) and *Bacteroidetes* phyla (17%). Consistent with the LEfSe analysis at the genus level, that found *Blautia* and *Ruminococcus* as the two

most significant differential genera by PSC status, we found that all 16 OTUs of *Blautia* genus were reduced while 4 of 5 OTUs of *Ruminococcus* genus and *Ruminococcaceae* family were enriched in PSC samples.

Correlation between microbiota genera and nutrients

Despite no significant differences were found in the daily intake of macro and micronutrients among groups, correlations between certain taxa and dietary components were disease specific. In IBD, *Dialister* (p=0.00028, r:0.93), and unknow genus from *Corioabacteriaceae* correlated with complex carbohydrate intake (p=0.00062, r=0.92), and *Veillonella* correlated with saturated fatty acids [(*eicosanoic acid,* p=0.044, r=0.84); (docosanoic *acid, p*=0.043, r=0.84). In PSC-IBD, *SMB53* genus was correlated with alcohol intake (p=0.012, r=0.87), *Bifidobacterium* with omega-6 (p=0.044, r=0.84), *f_Enterobactericae* with Boron (p=0.0025, r=0.90), and *Parabacteroides* with manganese (p=0.0071, r=0.88), and Selenium intake (p=0.0067, r=0.88). These results further support the already known modulating effect of diet on microbiota.

Correlation between microbiota genera and stool bile acids

Correlations between microbiota genus and the stool BA were calculated as described in our method section to test the interactions between gut microbiota and stool BA. Without stratifying by PSC status, we found four genera, including *Blautia* and *Veillonella* to be correlated to specific types of bile acids. In PSC-IBD, bacteria with significant correlations with BA metabolites mostly belonged to the *Firmicutes* phylum, specifically within the *Clostridia* and *Bacilli* classes. Different correlations were observed in IBD (**Table 10**).

Genus	BAs	Correlation[1]	Correlation[2]	p[1]	p[2]
All samples					
Blautia	TLCA	0.71	0.46	2.07E-02	1.78E-02
Collinsella	GDCA	0.69	0.45	4.66E-02	1.96E-02
Sutterella	CA	0.9	0.4	2.70E-07	4.18E-02

Veillonella	Ratio primary/ secondary	0.81	0.4	3.00E-04	4.52E-02
IBD samples					
SMB53	GCDCA	0.88	0.56	3.00E-02	4.56E-02
Megasphaera	GLCA-3S	0.96	0.56	6.39E-05	4.72E-02
PSC samples					
Coriobacteriaceae.g	TCDCA	0.88	0.9	4.06E-02	3.32E-05
Megamonas	GDCA	0.87	0.81	2.40E-02	3.87E-04
Veillonella	Sulfo-conjugates	0.96	0.8	5.05E-05	1.14E-03
Alistipes	GDCA	0.95	0.77	3.42E-04	1.87E-03
Veillonella	CDCA-3S	0.94	0.76	5.87E-04	2.62E-03
Roseburia	GDCA	0.89	0.73	2.85E-02	4.37E-03
Lactococcus	TUDCA	0.97	0.68	1.46E-05	1.03E-02
Bifidobacterium	TUDCA	0.9	0.63	1.27E-02	2.13E-02
Clostridiaceae.g	TLCA	0.99	0.63	6.60E-08	2.13E-02
Lachnospira	TLCA	0.95	0.63	2.66E-04	2.13E-02
Clostridiaceae.g	Glyco-conjugates	0.92	0.64	4.19E-03	2.23E-02
Veillonella	Total-Secondary BA	-0.91	-0.62	9.06E-03	2.26E-02
Veillonella	UDCA.3S	1	0.61	1.40E-11	2.78E-02
Veillonella	Ratio Primary/ secondary	0.9	0.6	1.06E-02	3.09E-02
Veillonella	Total Primary BA	0.92	0.59	4.05E-03	3.34E-02

Coprococcus	GDCA	0.94	0.58	1.09E-03	3.88E-02
Coprococcus	GCA	0.9	0.57	1.25E-02	4.73E-02
Streptococcus	CDCA	0.97	0.56	1.08E-05	4.86E-02

Table 10– The genus-BA pairs with both significant Pearson's correlation (adjusted p<0.05) and strong Spearman's correlations (p<0.05) are listed. P values were calculated using two tailed tests.

Compared to IBD, seven genera appeared and two genera disappeared in PSC-IBD. The total relative abundance of genera correlated to BA was 12% in PSC-IBD, compared to 0.4% in IBD. Two *Firmicutes*, *Lachnospira* and *Veillonella*, which were significantly reduced in PSC-IBD, showed strong correlations with multiple BA, only in PSC-IBD.

DISCUSSION

Herein, for the first time we have analyzed the stool BA profiles and their correlation with the fecal microbiota composition in patients with PSC-IBD as compared to IBD alone. The serum BA pool was increased and the stool BA pool was significantly reduced in PSC-IBD as compared to IBD alone. No significant differences in the individual stool bile acid components were found, but their overall composition differed from IBD (**Figure 17**). A significantly different microbiota composition based on the unweighted UniFrac distances was found between IBD and PSC-IBD, indicating differences in taxon composition for rare taxa (**Figure 18 A**). Specifically, PSC-IBD patients presented an enrichment in bacteria belonging to the genera *Ruminococcus* and *Fusobacterium* as compared to IBD alone (**Figure 18 A)**. Finally, specific microbiota-stool BA correlations were observed in PSC-IBD (**Table 10**).

In the past, some authors have hypothesized that the increased risk of right-sided colorectal neoplasia in PSC-IBD could be linked to an increase in secondary BA, although this had never been demonstrated[155, 264]. Normally, most of the bile acids (BA) secreted by the liver are efficiently reabsorbed in the terminal ileum, through the sodium-dependent BA transporter (ASBT), leaving only approximately 5% of the total BA to reach the colonic

lumen. In the right colon, primary BA are transformed in secondary BA mostly by bacterial mediated deconjugation, oxidation/reduction, epimerization and de-hydroxylation [265]. Therefore, fecal BA are mainly deconjugated, secondary BA. A small fraction of secondary BA is passively absorbed through the colonic mucosa, whilst the rest will be extruded with faeces[240]. During obstructive cholestasis, the expression of the apical BA transporter, which permits intracellular absorption of BA, is down-regulated, as a compensatory mechanism[236]. This could hypothetically lead to a relative increase in the proportion of BA entering the proximal colon in PSC-IBD patients, where they would be converted from primary into secondary BA. Interestingly, secondary BA have been shown to have anti-inflammatory properties but at the same have been shown to bear carcinogenic properties[256, 266-269]. Herein, we observed a significant reduction in the total stool BA in PSC-IBD as compared to IBD, which was expected taking into consideration the obstructive cholestatic nature of PSC. However, we did not find an increase in the relative proportion of the stool secondary BA in PSC-IBD patients, as previously hypothesized[264, 270]. No significant differences in individual proportion of serum or stool BA were found, which could perhaps be due to the small sample size. The proportion of DCA, a secondary BA was increased in PSC-IBD, although this did not reach statistical significance. Furthermore, the proportion of conjugate BA was also non-significantly increased in PSC-IBD as compared to IBD, which could indirectly indicate a decrease in the deconjugation activity of the microbiota, as previously observed in liver cirrhosis[206]. The decrease in *Bacteroides*, *Clostridium, Bifidobacterium*, and *Lactobacillus* genus, observed at the OTU level, bacteria known to be involved in bile acid deconjugation, could hypothetically be involved in this finding[271]. In this cohort, patients with PSC-IBD demonstrated an enrichment in bacteria from the *Ruminococcus* and *Fusobacterium* taxa, and a decrease in bacteria from the genus *Dorea*, *Veillonella*, *Lachnospira*, *Blautia*, and *Roseburia*. At the OTU level most shifts were observed within the *Firmicutes* (73%) and *Bacteroidetes* phyla (17%). Some of our findings are in consonance with recently published results on PSC microbiota also showing an increase of *Fusobacterium*[272] *(*a bacterial taxon that has been linked with adenomas and colorectal cancer) and in *Ruminococcus* in stool from patients with PSC-IBD or a decrease in *Roseburia* genus. However, as opposed to our findings, Kummen *et al.* reported PSC patients to have a significant increase in *Veillonella* genus in comparison to healthy controls and patients with IBD. In this cohort, *Veillonella* genus was positively correlated with disease severity and was more abundant in patients that had undergone OLT. Indeed, this genus has been reported to be increased in fibrotic

conditions such as liver or lung fibrosis or cystic fibrosis[273-276] In cirrhosis, *Veillonella* has also been associated with complications such as hepatic encephalopathy[277]. In another large cohort of patients with PSC-IBD, *Veillonella* was only significantly elevated in patients with PSC that presented concomitant liver cirrhosis.(7) Of note, in our PSC population, only 2 patients presented early liver cirrhosis, no patient presented severe PSC as measured by the PSC Mayo score or had undergone liver transplant. In our prior work we observed that *Blautia* was increased in the mucosa from PSC-IBD patients as compared to healthy controls[270]. This could be due to small sample size, and different populations with different geographic background and likely different diet habits. Furthermore, we cannot exclude the impact of different medications, disease severity etc in these contrasting findings, since the small sample sizes does not allow us to control for all these factors. Also worthy of note is the well described disconnect between mucosa and stool microbiota, as in prior work in liver cirrhosis, the stool and mucosa microbiome from the same individuals was also found to be distinct.[278]

The most important message from our work however, is that patients with PSC-IBD present distinct fecal microbiome-fecal BA correlation and interactions. No study had yet looked at the correlations between stool bile acid and the stool microbiota in PSC-IBD. However, BA pool size and composition have been shown to be important factors in regulating the gut microbiota[279-281]. Herein, despite our relatively small sample size, and after correcting for multiple comparisons, we were able to observe unique correlations between stool microbiota and stool bile acids in PSC-IBD. Within IBD alone, this broad BA-microbiota correlation disappeared. In particular, in PSC-IBD, the taxa that significantly correlated to the stool BA corresponded to ~12% of the total microbiota, while in IBD, this was less than 1%. Without any functional data, we may only speculate that these results suggest that under PSC condition, the BA changes may have dominant effects on defining the gut microbiota shifts, potentially towards a more pro-carcinogenic profile. Interestingly, bacteria from both *Fusobacterium* and *Ruminococcus* genus, are known to be involved in oxidation, epimerization and desulfatation of bile acids[271].

The major limitation of this study is our small sample size, which prevented us from adjusting for potential confounders in the microbiota and BA analysis. To overcome this, we tried to make our cohort as uniform as possible. All patients had pancolitis, and no patient had prior abdominal surgery or history of liver transplantation; all patients had mild to moderate PSC, as measured by the Mayo score, and dietary intake was also similar within

groups as assessed by the food frequency questionnaire. Furthermore, all patients stopped UDCA intake for 2 weeks and had no antibiotics or bowel preparation within at least 3 months of sample collection, all external factors that could potentially impact microbiota composition. While it may be argued that a 2-week interval to stop UDCA may not be enough to remove its effects, we did not observe any differences in the fecal bile acid composition or in the microbiota composition between those who were medicated with UDCA as compared to those who were not, consistent with what has been previously reported(7).

In summary, in this exploratory study, patients with PSC-IBD had a distinct stool bile acid and stool microbiota composition, as well as specific microbiota-stool bile acid correlations when compared to IBD. Whether these changes are associated with or may predispose to the specific PSC-IBD phenotype including the increased risk of colorectal neoplasia needs to be further clarified and warrants further research.

CHAPTER 5

HIGH RISK OF ADVANCED COLORECTAL NEOPLASIA AND ACCELERATED DYSPLASIA-CARCINOMA SEQUENCE IN PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

INTRODUCTION

Data on the risk of colorectal dysplasia and cancer (collectively termed as colorectal neoplasia - CRN) in patients with PSC were conflicting for some years. Studies with small sample sizes, different end points, and different comparison groups, as well problems in determining time of onset of IBD in patients with PSC, gave rise to disparate results. Broomé et al. in 1992 were the first to suggest that patients with IBD and PSC could have an increased risk for developing CRN [282]. Three years later, the same group showed that the absolute cumulative risk of developing CRN in PSC-UC patients after 10, 20, and 25 years of disease duration was 9%, 31%, and 50%, respectively, compared to 2%, 5% and 10% in those with UC alone ($p<0.001$)[283]. In this landmark study, 40 PSC patients with extensive UC who had been enrolled in a surveillance programme were matched to two control patients of the same age, also with extensive colitis and a comparable duration of disease but without PSC. Among the 40 PSC patients with UC, 16 developed CRN, versus only 10 out of 80 in the control group ($p< 0.001$).

These observations have since been reproduced in other studies[284-286]. By now, many publications have confirmed the increased risk of CRN in PSC-IBD patients, even when controlling for location and extent of disease[114, 119, 122, 284, 286-290], although in some series this increase in risk appeared to be low or even absent[64, 291-295]. A large recently published meta-analysis evaluating 13,379 patients with IBD, 1,022 (7.63%) of whom had concomitant PSC, showed that there was a three-fold increased risk of CRN and cancer among patients with PSC-IBD compared to the IBD-only population (odds ratio (OR) 3.24 [95% CI 2.14-4.90])[123]. Most importantly, Navaneethan *et al.* suggested a higher risk in the first two years after diagnosis of PSC-UC, but did not find any increased risk in the subsequent years, which decreases the likelihood that a longer disease course would increase the CRN risk[296]. There seems to be common features of CRN in PSC-IBD patients: extensive colon involvement[297], more frequent CRN in the right colon (proximal to the splenic flexure)[64, 117, 118, 122, 248, 298, 299]; and more frequent bile duct dominant stenosis (i.e. extrahepatic bile duct high-grade stenosis with obstruction)[300].

Given that the risks of CRN have been widely described in the PSC-IBD population, the different gastroenterology societies have commented on recommendations for surveillance in this group. Current recommendations support the use of annual colonoscopy and biopsies

in PSC-IBD patients from the time of PSC diagnosis, without taking into account the duration of IBD since it is often not known[301, 302].

However, we must acknowledge that prior studies had been performed in relatively small series of patients (due to the rarity of disease) and all studies report to an era when endoscopic enhanced surveillance (white light high definition scopes and/or chromoendoscopy) were still not used. Recent studies have shown that the risk of colorectal cancer and dysplasia in IBD seems to be decreasing. A large Danish population-based study including data from the National Patient Registry (NPR) and the Danish Cancer Registry during a 30-year follow-up period (1977- 2008) revealed no overall increase in risk of CRC in patients with IBD, except for some sub-groups. This trend, has been attributed to better control of inflammation, better implementation of colonoscopic surveillance and better endoscopic surveillance techniques, increased implementation of colectomy in some countries, and possibly chemopreventive effect the medications used for the treatment of IBD. Historically, random biopsies would be performed in the 4-quadrant and with 10 cm interval. The rationale for this was that most dysplasia was not visible. However, the advent of high definition endoscopes and chromoendoscopy techniques has changed this paradigm, and nowadays, targeted biopsies are being increasingly used, as it is believed that dysplasia is now in most cases visible. However, how and if these data apply the sub-group of patients with PSC-IBD is unknown.

The development of neoplasia in IBD colitis follows a multistep sequence from chronic inflammation and no dysplasia or indefinite dysplasia (IND) to low grade-dysplasia (LGD) and high-grade dysplasia (HGD) prior to final malignant transformation to adenocarcinoma. As such, the presence and grade of dysplasia remain the best current indicators of cancer risk in IBD. Despite providing estimate rates on advanced colorectal neoplasia (aCRN) in patients with PSC-IBD, prior studies had failed to study the natural history of lower grades of dysplasia (such as indefinite or low-grade dysplasia) to more advanced neoplasia (high-grade dysplasia or colorectal cancer). Patients with HGD have a high risk of developing cancer, and therefore colectomy is recommended in those cases that cannot be managed endoscopically. The management of IND and LGD dysplasia is however more controversial, because of the heterogeneous reported rates of progression to cancer varying between 16-54%, and while some centers may choose to follow a tighter surveillance protocol, others may recommend immediate colectomy. Overall, there is an increasing tendency to keep IBD

patients with LGD on intensive surveillance instead of recommending proctocolectomy. [1819] However, while some studies have described the rate of progression of indefinite and LGD in IBD-only patients, the rate of progression of colorectal neoplasia in patients with PSC-IBD has not been thoroughly studied, and the fate of IND and LGD and the rate of progression of IND and LGD to more advanced neoplasia in PSC-IBD patients are not well characterized. One would hypothesize that the rate of progression of IND/LGD to HGD or CRC is faster than in patients with UC alone, but this has never been confirmed. A small study on 10 patients with PC-IBD and LGD reported a rate of progression of LGD to HGD/CRC in 30% of patients over a follow-up period of 13±11months[303]. In an earlier study including IBD patients with LGD, PSC was the only risk factor for increased risk of progression from LGD to HGD/CRN[304].

RATIONAL AND AIMS FOR THIS STUDY

Rational: Despite the well accepted risk of colorectal neoplasia in PSC-IBD, data comes predominantly from older studies. Whether the same decrease in the rates of aCRN are still seen in these patients remains to be described in a modern cohort. Additionally, no specific recommendations exist on the management and follow-up of indefinite or LGD exist for this high-risk population that may be more prone to be referred for colectomy, given the elevated risk of CRC. One would hypothesize that once indefinite or low-grade dysplasia (LGD) is diagnosed in PSC-IBD, the rate of progression to high-grade dysplasia or CRC would be faster than in IBD patients. Hence, further studies are needed to better determine the outcomes regarding low-grade and indefinite dysplasia in PSC-IBD patients.

Aim: Our aim was to verify whether in this era of improved medical treatment and improved surveillance, patients with PSC-IBD will still have higher proportion of advanced colorectal neoplasia overall, and to assess the rates of aCRN development following the diagnosis of Indefinite dysplasia or low-grade dysplasia.

Approach: A large multicenter international retrospective cohort, with longitudinal data, comprising patients with a confirmed diagnosis of PSC and established colonic IBD undergoing colonoscopic surveillance for neoplasia between 2000-2015 from 2 secondary and 6 tertiary centers from the Netherlands and one tertiary center from Mount Sinai Hospital in New York City was assembled. Patients were carefully characterized and clinical,

endoscopic, and histological information was retrieved at every colonoscopy during follow-up and documented according to a predefined protocol using the same database. The incidence rates of aCRN overall and of aCRN following a diagnosis of IND and LGD will be compared.

Contribution of the PhD candidate

The candidate was the responsible for the research idea and concept, after discussion with Prof. Marília Cravo, Prof Jean-Frédéric Colombel and Dr. Steven H. Itzkowitz. To accomplish this project, the candidate wrote the protocol and created the electronic database in the REDCAP software to capture all relevant information, and created the search query which was used to identify patients within the Mount Sinai Health System Data warehouse. The candidate contributed to data abstraction and data capture alongside with junior colleagues who showed interest in participating in this research. Later on, after a meeting with Dutch investigators where preliminary results were presented, the decision was made to combine both databases. The candidate participated in this process and contributed to the framework of the statistical analysis in collaboration with Dr. Joren tenHove in the Netherlands. The candidate was involved in the planning and writing of the paper in collaboration with Dr. Sha and Dr. tenHove. This fruitful collaboration resulted in the successful presentation of the work below in several international conferences, and finally in the publication of this work in Clinical Gastroenterology and Hepatology (**Impact factor**: **7.39):** "***High Risk of Advanced Colorectal Neoplasia in Patients with Primary Sclerosing Cholangitis Associated with Inflammatory Bowel Disease.*** *Shah SC, Ten Hove JR, Castaneda D, Palmela C, Mooiweer E, Colombel JF, Harpaz N, Ullman TA, van Bodegraven AA, Jansen JM, Mahmmod N, van der Meulen-de Jong AE, Ponsioen CY, van der Woude CJ, Oldenburg B, Itzkowitz SH, Torres J. Clin Gastroenterol Hepatol. 2018 Mar 14. pii: S1542-3565(18)30089-2. doi: 10.1016/j.cgh.2018.01.023. [Epub ahead of print]"*

MATERIAL AND METHODS

Study population and case identification

Patients with established IBD colitis undergoing colonoscopic surveillance between 2000-2015 were retrospectively identified from two databases: A Dutch database inclusive of 2 secondary and 6 tertiary centers and the Mount Sinai Hospital database in New York City inclusive of one tertiary IBD referral center. Cases were identified by query of the electronic health record (EHR)-linked database utilizing both ICD-9 and -10 codes and free text searches for cases of IBD and free text searches for PSC.

Patient selection (inclusion and exclusion criteria)

After initial identification through the EHR query, individual charts were reviewed. For PSC-IBD patients, a clinical diagnosis of PSC had to be confirmed by distinctive features on cholangiography or liver biopsy (for those with small-duct PSC). Additional inclusion criteria were: 1) diagnosis of IBD [UC, CD, IBD unclassified (IBD-U)] with colonic involvement confirmed endoscopically and histologically; 2) confirmed colonic disease duration of at least 8 years for non-PSC IBD or any colonic disease duration for PSC-IBD patients; 3) enrollment in a surveillance program and 4) at least left-sided colitis (UC or IBD-U) or involvement of >30% of the colonic surface (CD or IBD-U). Patients with a history of colectomy prior to enrollment or history of aCRN prior to or at the index colonoscopy during the defined study period were excluded. Surveillance procedures were defined as colonoscopies in which either segmental random biopsies or chromoendoscopy were employed. Colonoscopies with other indications, e.g. medically refractory disease, were excluded. The index colonoscopy was defined as the first surveillance colonoscopy performed within the study period (2000-2015).

Data collection

Database coding was identical for all study populations. The date of study entry was set at the first surveillance colonoscopy in the database. The time of onset of PSC or IBD was determined from EHR review. The date of the last colonoscopy was set as the last day of follow-up.

The following baseline demographic and clinical data were abstracted: date of birth, sex, date of PSC diagnosis (if applicable), date of IBD diagnosis, IBD type, maximum disease extent, and date of prior diagnosis of IND and/or LGD (if applicable). Maximum disease extent was defined as the maximum documented extent of endoscopic disease on any colonoscopy and was coded as follows: extensive/pancolitis (>50%) or intermediate/left-sided (30-50%). Medication exposure (at least one prescription) was recorded for 5-aminosalicylates (5-ASA), thiopurines, and biologics.

Data from each surveillance colonoscopy was recorded, including date of exam, quality of bowel preparation (adequate or inadequate), most proximal extent examined, use of chromoendoscopy, presence and severity of endoscopic inflammation, presence of post-inflammatory polyps ("pseudopolyps"), stricture(s) and visible lesions. Endoscopically detected neoplastic lesions were categorized based on morphology (polypoid/non-polypoid). Endoscopically invisible neoplasia was defined as neoplasia detected in a random biopsy with no corresponding morphologic lesion seen on endoscopy. Right-sided lesions were defined as those proximal to the splenic flexure. For each surveillance colonoscopy, severity of active endoscopic inflammation was scored on a 4-point scale for each colonic segment visualized: 0 (no inflammation/remission), 1 (mild inflammation), 2 (moderate inflammation) or 3 (severe inflammation). A mean inflammatory severity score per patient and per colonoscopy was calculated by dividing the sum of inflammatory severity scores by the total number of colonic segments visualized per colonoscopy and then by the total number of surveillance colonoscopies.

Histology

Dysplasia was recorded as indefinite (IND), low-grade (LGD), or high-grade (HGD). All histologic diagnoses were as reported in the original pathology report; no specimens were re-reviewed or altered for this study. Of note, it is routine clinical practice at each participating institution that all pathology concerning for colorectal neoplasia is reviewed at the time of diagnosis and agreed upon by at least two pathologists.

Primary and secondary outcomes

The primary outcome was a diagnosis of aCRN, defined as HGD or CRC, during follow-up. Secondary outcomes were a diagnosis of IND and/or LGD during follow-up and the

development of aCRN following a diagnosis of IND and/or LGD. Factors associated with a diagnosis of aCRN in both PSC-IBD and non-PSC IBD patients with or without a prior diagnosis of IND and/or LGD were explored.

Statistical analysis

Basic descriptive statistics were generated for patients meeting inclusion criteria. Chi-square and Fisher's exact tests were used to compare categorical variables and dichotomous outcomes, while the Student's t-test and Mann-Whitney U-test were used for analyzing continuous data. Incidence rates were calculated as the number of cases per 100 patient-years (pty) of follow-up. Univariate and multivariate Cox-regression modeling was used to identify factors associated with aCRN. The proportional hazards assumption of time-static covariates was assessed using log-log plots and Schoenfeld residuals. Because inflammatory scores were not stable over time, these were inputted as time-changing covariates into the models. Mean inflammation scores were re-calculated at every time-point for each patient to correct for the also variable number of colonoscopies. A p-value of ≤ 0.10 was used as the cutoff for selecting variables for the multivariate analysis. Kaplan-Meier survival curves were generated to compare cumulative incidence rates. Follow-up data were censored at the last point of colonoscopic follow-up, aCRN diagnosis, or colectomy. All data analyses were performed using SPSS version 22 (Armonk, NY: IBM Corp.).

RESULTS

Baseline demographic and clinical characteristics

Of 1,911 patients with colonic IBD in the combined database meeting inclusion criteria, 293 patients were confirmed to have PSC-IBD; the remaining 1,618 patients with non-PSC IBD served as the comparison group (**Figure 20**).

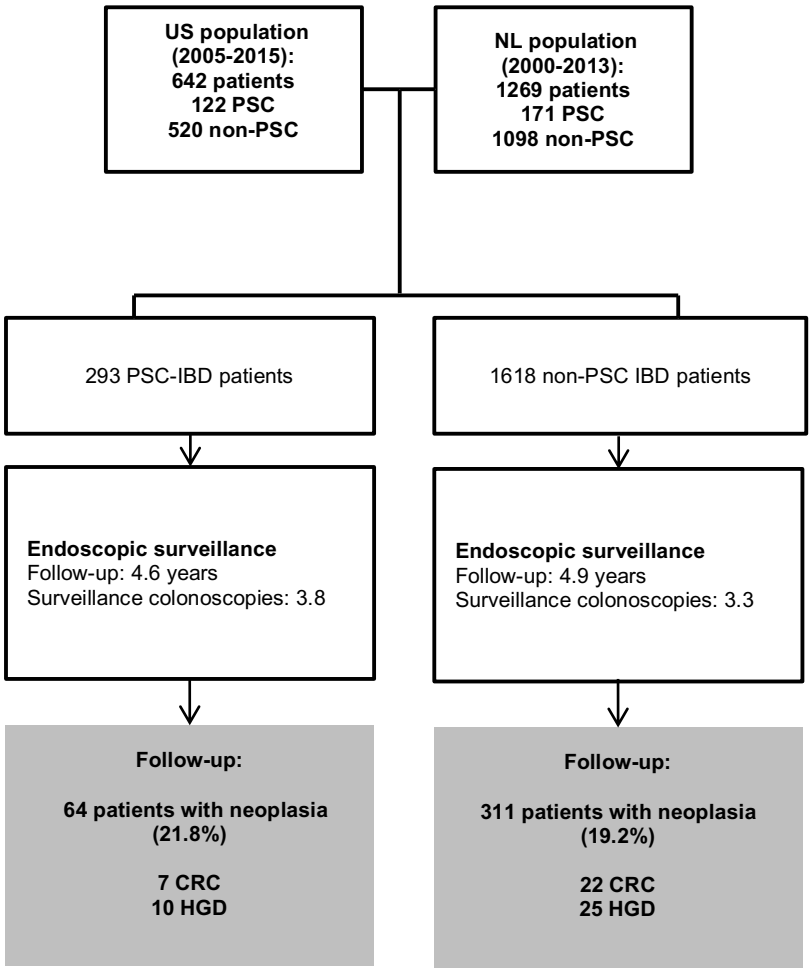


Figure 20 - Description of patient selection and main outcomes in each database. Overall, 293 PSC-IBD patients were compared with 1618 IBD alone patients. The man follow-up period for the whole cohort was 4.8 years.

The main demographic and clinical features of the cohort are detailed in **Table 11**.

	PSC-IBD (n=293)	**Non-PSC IBD (n=1618)**	**p-value**
Male (%)	205 (70.0%)	796 (49.2%)	<0.001
Age at study inclusion, mean (SD)	39 (14)	45 (13)	<0.001
IBD type **- Ulcerative colitis** **- Crohn's colitis**	 203 (69.3%) 76 (25.9%)	 912 (56.4%) 661 (40.9%)	 <0.001

- Indeterminate colitis	14 (4.8%)	45 (2.8%)	
Disease extent			
- Not specified	34 (11.8%)	154 (9.6%)	
- Limited extent/proctitis	13 (4.5%)	49 (3.1%)	<0.001
- Intermediate/Left-sided	41 (14.2%)	572 (35.8%)	
- Extensive/pancolitis	201 (69.6%)	823 (51.5%)	
Age at IBD diagnosis, mean (SD)	27 (13)	28 (12)	0.11
IBD duration, mean (SD)	12 (10)	17 (9)	<0.001
Age at PSC diagnosis, mean (SD)	32 (14)	-	-
Medication use			
- 5-ASA	221 (75.4%)	1316 (81.3%)	0.02
- Thiopurines	93 (31.7%)	825 (51.0%)	<0.001
- Biologicals	38 (13.0%)	402 (24.8%)	<0.001
Duration of follow-up after index colonoscopy			
- mean (SD)	4.6 (3.2)	4.9 (3.0)	0.10
- median	4.1	4.5	
Number of surveillance colonoscopies (mean)	3.8	3.3	<0.001

Table 11 - Baseline characteristics of the study population. PSC: Primary sclerosing cholangitis; IBD: inflammatory bowel disease.

Compared with the non-PSC IBD group, PSC-IBD patients were more often male and younger at study entry, although the age of IBD diagnosis was similar between groups (p=0.11), and thus reflecting earlier surveillance start in the PSC-IBD group. Likewise, the IBD disease duration was shorter in the PSC group. As expected, UC was the predominant IBD type in the PSC-IBD group. PSC-IBD patients were less frequently exposed to IBD therapy compared to non-PSC IBD patients, possibly reflecting less clinically active disease.

In 151 patients (51.5%), the PSC diagnosis was established after the IBD diagnosis, while in 36 (12.3%) PSC was established before the IBD diagnosis. For the remainder, PSC and IBD were diagnosed within the same year or the sequence of diagnoses was not recorded. The mean follow-up for the total cohort was 4.8 (±3.0) years, with a total of 9,265 patient-years of follow-up; there was no difference in follow-up time between PSC-IBD and non-PSC IBD patients. The number of surveillance colonoscopies performed within the study period was higher in PSC-IBD patients (3.8 vs. 3.3, p<0.01).

Inflammatory activity

The endoscopic severity of inflammation on surveillance exams was similar between PSC-IBD and non-PSC IBD patients (**Table 12**). As expected, the proportion of procedures in which extensive active disease was observed in PSC-IBD vs. non-PSC IBD patients was 27% vs. 12% (p<0.01), 23% vs. 10% (p<0.01), and 27% vs. 10% (p<0.01) for the first, second and third surveillance colonoscopy, respectively. The proportion of patients in endoscopic remission on each of their surveillance colonoscopies during the entire study period was higher in non-PSC IBD compared to PSC-IBD patients (p=0.02).

	PSC-IBD	**Non-PSC IBD**	**p-value**
Severity of active inflammation, mean [0-3]*	0.55	0.56	0.89
Extent of active inflammation, mean [0-3]*	1.36	1.17	0.003
Activity ratio for all surveillance colonoscopies (active/inactive)	45%	41%	0.19
No inflammation on ALL surveillance colonoscopies	76 (27.1%)	546 (34.1%)	0.02
Inflammation extent (1st colonoscopy) **- No activity** **- Limited** **- Intermediate** **- Extensive/pancolitis**	 127 (53.6%) 7 (3.0%) 38 (16.0%) 65 (27.4%)	 864 (57.9%) 89 (6.0%) 363 (24.3%) 176 (11.8%)	0.001
Inflammation extent (2nd colonoscopy) **- No activity**	 125 (55.3%)	 866 (61.2%)	

- Limited	9 (4.0%)	109 (7.7%)	<0.001
- Intermediate	40 (17.7%)	297 (21.0%)	
- Extensive/pancolitis	52 (23.0%)	141 (10.0%)	
Inflammation extent (3rd colonoscopy)			
- No activity	102 (57.3%)	584 (63.6%)	<0.001
- Limited	6 (3.4%)	79 (8.6%)	
- Intermediate	22 (12.4%)	164 (17.9%)	
- Extensive/pancolitis	48 (27.0%)	92 (9.9%)	
Endoscopic inflammation severity (1st colonoscopy)			
- No activity	160 (57.1%)	924 (57.8%)	0.20
- Mild	100 (35.7%)	495 (30.9%)	
- Moderate	19 (6.8%)	131 (8.2%)	
- Severe	1 (0.4%)	50 (3.1%)	
Endoscopic inflammation severity (2nd colonoscopy)			
- No activity	125 (53.6%)	864 (59.3%)	0.77
- Mild	89 (38.2%)	445 (30.5%)	
- Moderate	16 (6.9%)	101 (6.9%)	
- Severe	3 (1.3%)	48 (3.3%)	
Endoscopic inflammation severity (3rd colonoscopy)			
- No activity	102 (55.4%)	583 (61.6%)	0.17
- Mild	63 (34.2%)	276 (29.2%)	
- Moderate	13 (7.1%)	64 (6.8%)	
- Severe	6 (3.3%)	23 (2.4%)	

Table 12 - Inflammatory parameters during surveillance. *=corrected for total number of surveillance colonoscopies per patient

Occurrence of aCRN and associated risk factors

Among PSC-IBD patients, aCRN was diagnosed in 17 patients (5.8%), with CRC in 7 (2.4%) and HGD in 10 patients (3.4%) (**Table 13**).

	PSC-IBD (n=293)	Non-PSC IBD (n=1618)	p-value
Advanced neoplasia (aCRN)	17 (5.8%)	47 (2.9%)	0.01
- CRC	7 (2.4%)	22 (1.4%)	0.19
- HGD	10 (3.4%)	25 (1.5%)	0.03
LGD (patients with ≥1 LGD lesion)	60 (20.5%)	295 (18.2%)	0.37
IND (patients with IND as highest grade lesion)	27 (9.2%)	74 (4.6%)	0.001
Time from IBD diagnosis to aCRN diagnosis, mean (years)	19.4	24.3	0.15
Time from database entry to aCRN diagnosis, mean (years)	4.2	3.4	0.31
Time from LGD to aCRN diagnosis, mean (years)	0.7	1.7	0.12

Table 13 - Description of the outcomes during the study period. CRC=colorectal cancer, HGD=high-grade dysplasia, LGD=low-grade dysplasia, IND= indefinite dysplasia; AN=advanced neoplasia, y=years

The frequency of aCRN during follow-up was significantly lower in non-PSC IBD patients (2.9%), with CRC and HGD diagnosed in 1.4% and 1.5% patients respectively (p=0.01). The incidence rate of aCRN in PSC-IBD compared to non-PSC IBD patients was significantly higher (1.3 vs. 0.6/100pty, p<0.01) (**Figure 21**).

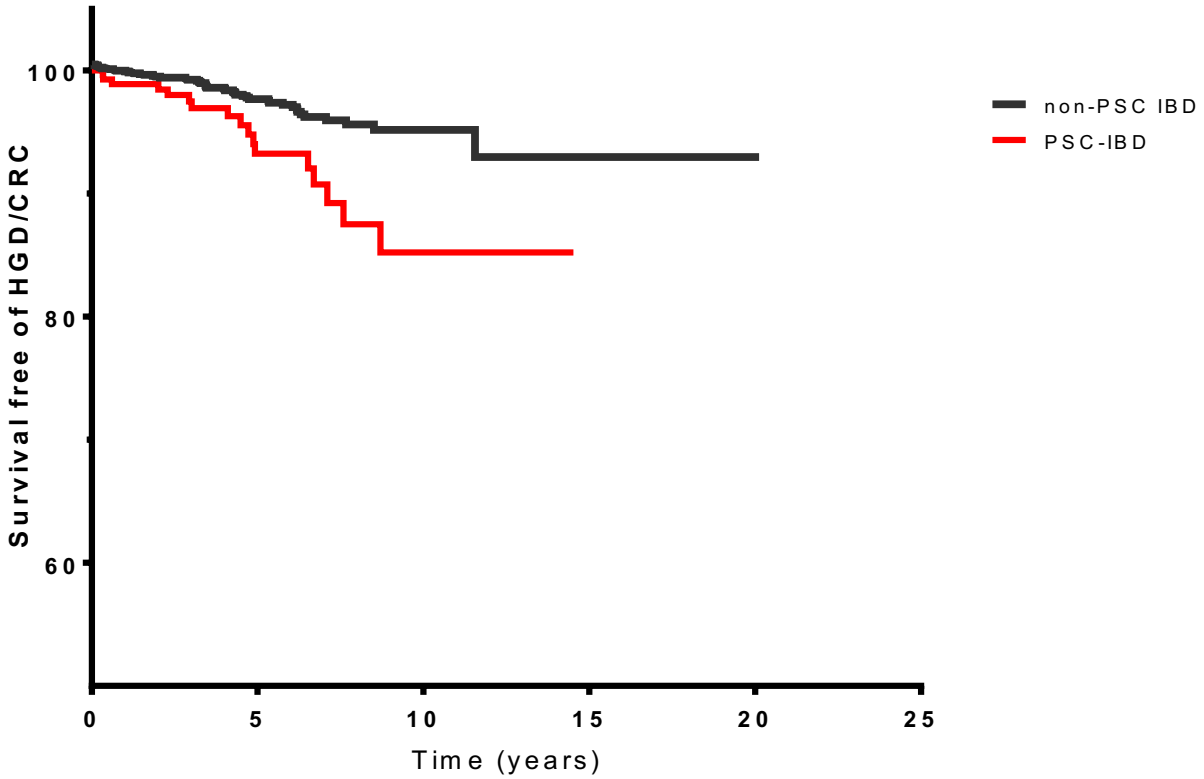


Figure 21 - Kaplan-Meier time-to-event (aCRN) analysis, all patients since study entry. The Incidence rates were calculated as the number of cases per 100 patient-years (pty) of follow-up. The incidence rate of aCRN in PSC-IBD compared to non-PSC IBD was 1.3 vs. 0.6 per 100 patients years (Log rank, $p<0.01$).

While aCRN was more often right-sided in PSC-IBD compared to non-PSC IBD patients, this was not statistically significant (53% vs. 31%, p=0.12). Among 40 PSC-IBD patients (14%) where the diagnosis of PSC was newly established within the study period, three cases of aCRN occurred, with a mean duration of 4.0 years (±2.5) between the PSC diagnosis and aCRN occurrence.

Since we were including patients from different geographic region and different medical settings (tertiary and secondary centers), we compared the main patient´s characteristics, as well the primary outcomes stratified by study site. Results can be found detailed in **Table 14.** As it can be seen, there were some differences between study sites, namely higher proportion of exams with high-grade and low-grade dysplasia in the US vas compared to the Netherlands, probably reflecting the tertiary center setting. No major differences in the patient population were found, namely in what regards proportion of extensive colitis/pancolitis, male gender or number of surveillance colonoscopies.

	NL (n=1269)	US (n=642)	p-value
Male (%)	674 (53.1%)	327 (50.9%)	0.37
Age at study inclusion, mean (SD)	45 (12)	41 (15)	<0.001
Age at IBD diagnosis, mean (SD)	29 (12)	26 (14)	<0.001
Primary sclerosing cholangitis (PSC)	171 (13.5%)	122 (19.0%)	0.002
Age at PSC diagnosis, mean (SD)	33 (12)	32 (16)	0.37
IBD type **- Ulcerative colitis** **- Crohn's colitis** **- Indeterminate colitis**	 800 (63.0%) 434 (34.2%) 35 (2.8%)	 315 (49.1%) 303 (47.2%) 24 (3.7%)	<0.001
Extensive disease / pancolitis	686 (54.1%)	338 (52.6%)	0.56
Medication use **- 5-Aminosalicylates** **- Thiopurines** **- Biologicals**	 999 (78.7%) 556 (43.8%) 156 (12.3%)	 543 (83.8%) 362 (56.4%) 284 (44.2%)	 0.008 <0.001 <0.001
Number of surveillance colonoscopies (mean)	3.4	3.3	0.25
Interval between surveillance colonoscopies, years (mean)	1.6	1.2	<0.001
Neoplasia Outcomes:			
Colorectal cancer (CRC)	17 (1.3%)	12 (1.9%)	0.37
High-grade dysplasia (HGD)	15 (1.2%)	20 (3.1%)	0.003
Low-grade dysplasia (LGD)	264 (20.8%)	88 (13.7%)	<0.001

Table 14: **Database characteristics: comparison Netherlands (NL) versus United States (US).**

We then sought to determine which were the independent risk factors for advanced colorectal neoplasia development. On multivariate Cox-regression analysis, PSC (adjusted HR (aHR) 2.01, 95%CI: 1.09-3.71), increasing age (aHR 1.03, 95% CI: 1.01-1.05), and active inflammation (aHR 2.39, 95%CI: 1.63-3.49) remained independent predictors of aCRN diagnosis during follow-up after adjusting for severity of inflammation over time, as time changing co-variate (**Table 15**).

	Univariate			**Multivariate**		
Variable	HR	95% CI	P-value	aHR	95% CI	P-value
Age (years)	1.02	1.01-1.04	0.03	1.03	1.01-1.05	0.007
Age at IBD diagnosis	1.00	0.98-1.02	0.78			
Sex (male)	1.83	1.08-3.08	0.02	1.62	1.94-2.79	0.08
PSC	2.13	1.22-3.70	0.01	2.01	1.09-3.71	0.03
Inflammation severity, mean [0-3]*	2.14	1.48-3.09	<0.001	2.39	1.63-3.49	<0.001
IBD type (reference: UC)	0.99	0.60-1.61	0.95	ns		
Maximum disease extent (reference: pancolitis)	1.43	0.85-2.41	0.18			
Thiopurine exposure	0.84	0.51-1.40	0.85			
Biological exposure	0.72	0.36-1.46	0.36			
5-ASA exposure	1.14	0.58-2.25	0.70			
Number of surveillance procedures	0.96	0.84-1.09	0.53			

Table 15: Uni- and multivariate Cox-regression analysis for the overall risk of aCRN (all patients). Listed are the Hazard ratios (HR) and corresponding p values. Non-significant values are not listed. *entered as time-changing covariate; 0 (no inflammation/remission), 1 (mild), 2 (moderate), 3 (severe)

Correcting for geography (US vs. Netherlands) did not affect these findings (**Table 16**).

	Univariate			Multivariate		
Variable	HR	95% CI	p-value	aHR	95% CI	p-value
PSC	2.13	1.22-3.70	0.008	1.85	1.00-3.43	0.049
Inflammation (severity [0-3])*	2.14	1.48-3.09	<0.001	2.08	1.42-3.07	<0.001
Sex (reference: male)	1.83	1.08-3.08	0.02	1.68	0.97-2.89	0.06
IBD type (reference: UC)	0.99	0.60-1.61	0.95			
Maximum disease extent (reference: pancolitis)	1.43	0.85-2.41	0.18			
Age at IBD diagnosis	1.00	0.98-1.02	0.78			
Age (years)	1.02	1.01-1.04	0.03	1.03	1.01-1.05	0.004
Thiopurine exposure	0.84	0.51-1.40	0.85			
Biological exposure	0.72	0.36-1.46	0.36			
5-aminosalicylate exposure	1.14	0.58-2.25	0.70			
Number of surveillance procedures	0.96	0.84-1.09	0.53			
Population (reference: US)	2.82	1.72-4.62	<0.001	2.20	1.30-3.74	0.003

Table 16 - Uni- and multivariate Cox-regression analysis for the overall risk of aCRN (all patients), corrected for study site. *entered as time-changing covariate; 0 (no inflammation/remission), 1 (mild), 2 (moderate), 3 (severe).

Risk of aCRN following a diagnosis of IND and/or LGD

The number of patients in the total cohort with at least one diagnosis of IND was 147 (7.7%). In 101 patients (5.3%) no additional dysplasia was detected. Among patients with a diagnosis of IND, the rate of developing aCRN following detection of IND was higher in PSC-IBD compared to non-PSC IBD patients (p=0.02) (**Figure 22**).

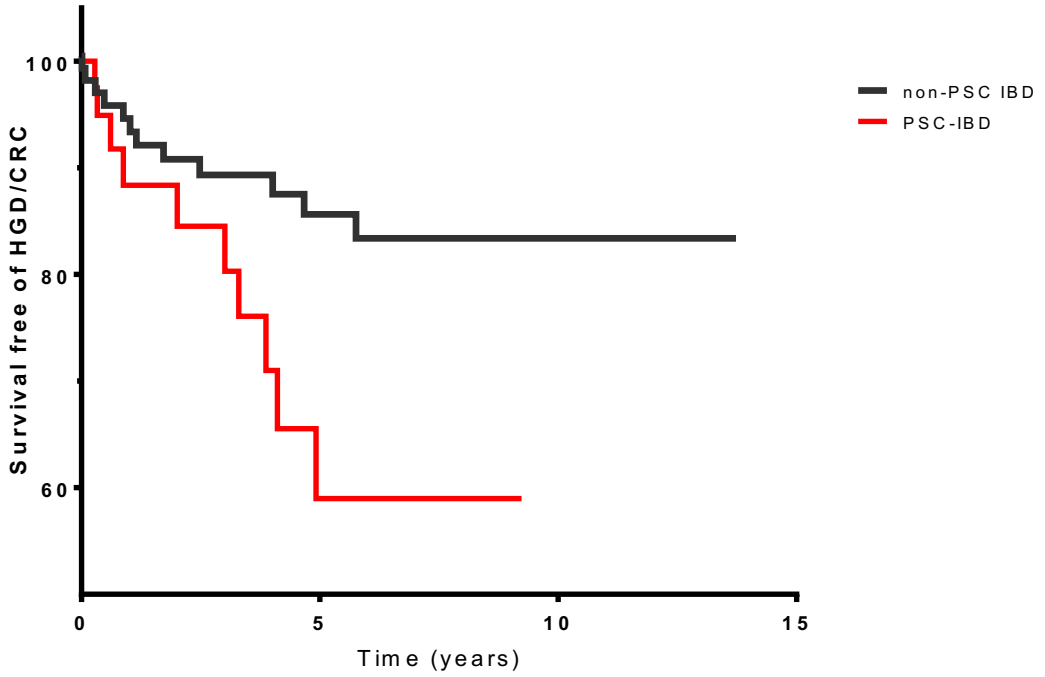


Figure 22 - Kaplan-Meier time-to-event (aCRN) analysis for patients with IND; time from first IND within study interval to event. (p=0.02, log-rank test). IND: indefinite dysplasia

However, when patients with a synchronous or metachronous diagnosis of LGD (n=46) were excluded from this analysis (i.e. no grade of dysplasia higher than IND), this difference was no longer significant.

The occurrence of at least one LGD-containing lesion during the study period was similar for both PSC-IBD and non-PSC IBD patients (21% vs. 18%, p=0.37). Despite a similar proportion of patients with LGD, the rate of developing aCRN following detection of LGD was almost 3-fold higher in PSC-IBD compared to non-PSC IBD patients (8.4 vs. 3.0/100pty, p=0.01 (**Figure 23**).

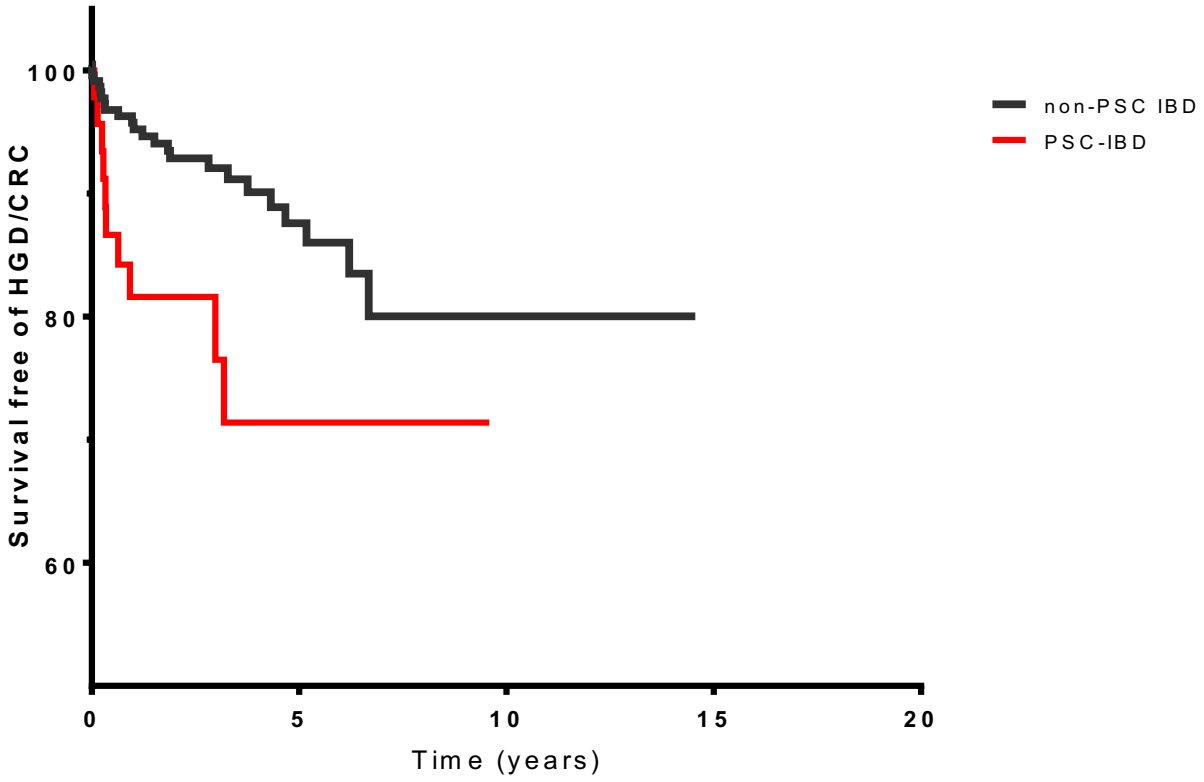


Figure 23 - Kaplan-Meier time-to-event (aCRN) analysis, patients with LGD only. The incidence rate of aCRN patients following a diagnosis of LGD was The incidence rate of aCRN in PSC-IBD compared to non-PSC IBD was 8.4 versus 3.0 per 100 patient years (Log rank, p<0.001)-

For the subgroup of patients with LGD, the number of patients in whom endoscopically invisible LGD was detected over the course of surveillance was higher in PSC-IBD patients (38% vs. 22%, p=0.01). The proportion of invisible LGD cases among the total number of LGD cases (per-colonoscopy analysis) was also higher. In a sub-analysis of the Netherlands population, we corrected for the total number of random biopsies taken (107,745 biopsies in total); the number of random biopsies needed to detect invisible dysplasia was 826 in PSC-IBD patients compared to 1,703 in non-PSC IBD patients.

On univariate Cox regression analysis, only PSC and multifocal dysplasia were associated with higher risk of aCRN diagnosis following LGD detection, while polypoid morphology of the lesion (vs. nonpolypoid or invisible) was associated with a lower risk. On multivariate analysis, only polypoid morphology remained significant and was associated with a reduced

risk of aCRN (aHR 0.31, 95%CI: 0.14-0.65) following LGD detection compared to nonpolypoid or endoscopically invisible lesions (**Table 17**).

	Univariate			**Multivariate**		
Variable	HR	95% CI	p-value	aHR	95% CI	p-value
PSC	2.52	1.19-5.31	0.02	1.79	0.83-3.88	0.14
Sex (reference: male)	1.23	0.60-2.49	0.57			
Thiopurine exposure	1.20	0.60-2.40	0.60			
Biological exposure	0.74	0.23-2.44	0.74			
5-Aminosalicylate exposure	1.07	0.44-2.59	0.88			
Dysplasia characteristics						
Distal location	1.69	0.77-4.32	0.17			
Multifocality	2.46	1.22-4.95	0.01	1.90	0.93-3.87	0.08
Polypoid morphology	0.27	0.13-0.57	0.001	0.31	0.14-0.65	0.002
Invisible dysplasia	1.64	0.76-3.53	0.21			
Nonpolypoid morphology	1.82	0.70-4.74	0.22			

Table 17- Uni- and multivariate Cox-regression analysis for the risk of aCRN* following detection of low-grade dysplasia (LGD). *advanced colorectal neoplasia, aCRN: defined as colorectal cancer and/or high-grade dysplasia

DISCUSSION

In the work herein presented through a collaboration developed with a Dutch group that also performs research on the field of dysplasia and IBD, we were able to assemble a large multicenter international retrospective cohort with longitudinal data. This cohort comprised patients with a confirmed diagnosis of PSC and established colonic IBD undergoing colonoscopic surveillance for neoplasia between 2000-2015 from 2 secondary and 6 tertiary centers from the Netherlands and one tertiary center from Mount Sinai Hospital in New York

City. Patients were carefully characterized and clinical, endoscopic, and histological information was retrieved at every colonoscopy during follow-up and documented according to a predefined protocol using the same database. Through this collaboration, we could include 1911 patients (293 PSC-IBD, 1618 non-PSC IBD) for a total of 9,265 patient-years of follow-up, making our study probably the largest cohort of PSC-IBD patients undergoing surveillance. Having access to such a large database with longitudinal information, we were not only able to corroborate previous literature reporting a higher risk of aCRN in patients with concomitant PSC as compared to those without PSC, but also add to the literature by confirming this in the current era of improved endoscopic technology and more effective medical therapy for inflammation. In our well-characterized surveillance cohort, which, to our knowledge, is the largest published cohort of PSC-IBD patients undergoing surveillance, we found that PSC-IBD is associated with a 2-fold higher risk of aCRN. This risk is slightly lower as compared to prior studies as a recent meta-analysis of 16 studies reported a 3.4-fold higher odds for colorectal neoplasia in PSC-IBD patients[305]. Importantly, the increased risk in our study remained after correcting for active endoscopic inflammation over time (which was employed as a time-changing covariate rather than a mean overall score). Endoscopic activity assessed during subsequent colonoscopies was strongly associated with the risk of future aCRN, congruent with studies in the non-PSC IBD population[306]. Whether the outcomes of endoscopic inflammation compared to histologic inflammation are distinct remains a question for future investigation.

We further expand knowledge in the field by reporting an even higher risk of aCRN following detection of LGD (but not IND alone). An important observation from our study that distinguishes PSC-IBD from non-PSC IBD patients, is that dysplasia was more often detected in random biopsies. While previous retrospective studies have shown a low overall yield for dysplasia with random biopsies as opposed to only targeted biopsies of visible lesions, there was higher yield for dysplasia on random biopsy in those with concurrent PSC. Our data further add to this body of evidence, and therefore it can be questioned whether the current recommendation based on the results of prospective studies to move away from random biopsies as part of CRC surveillance should be applied to PSC-IBD patients[307, 308] That LGD was more often endoscopically invisible in PSC-IBD compared to non-PSC IBD validates the more intensive management considerations for this population. During surveillance examinations, particular attention should be paid to the proximal colon as right-

sided cancers seem to be more common in PSC-IBD compared to non-PSC IBD colitis[155]. While the proportion of right-sided aCRN was higher in the PSC- subgroup, this difference was not statistically significant in the present study and may be due to insufficient power; it may also reflect selection bias since one of our inclusion criteria for the non-PSC IBD subgroup was at least left-sided disease extent or more than 30% involvement, and thus may not represent the overall IBD population. Our study confirms that the date of PSC diagnosis is particularly relevant when risk-stratifying patients, since it seems that the risk of neoplastic progression is highest within the first few years of the PSC diagnosis.[36] In a sub-analysis of newly diagnosed PSC-IBD patients within our study time frame, all cases of aCRN occurred within 8 years of PSC diagnosis. Thus, while CRC surveillance is recommended after disease duration of 8 years in patients with colonic IBD and no PSC, CRC surveillance at the time of diagnosis in the setting of PSC is recommended and further corroborated by our findings.

Our study has several strengths. In addition to being perhaps the largest IBD surveillance cohort in the modern era, our cohort is particularly robust since each patient was confirmed to have colonic IBD and to be actively enrolled in a colonoscopic CRC surveillance program. Comprehensive data on disease history and endoscopic findings during surveillance allowed for more accurate neoplastic risk assessment, particularly with respect to measurement of inflammatory burden over time. Importantly, detailed information on inflammatory activity at each colonoscopy was incorporated into the analysis for more accurate assessment of aCRN development in PSC-IBD. Our study also has some limitations, most notably the retrospective design. Although we combined surveillance cohorts from two different countries, we predefined the inclusion/exclusion criteria, variables to be assessed, and definitions of outcomes. Combining these two cohorts enhanced not only our power to detect meaningful differences, but also the generalizability of our findings given that our study population included patients from affiliated sites as well as tertiary IBD referral centers. Even so, while we did not detect striking differences in patient demographics or follow-up patterns between the two countries, there may be unmeasured differences in care pathways between the included sites. That said, our results remained significant even after adjusting for geography. Unfortunately, the lack of standardized guidelines for the use of chromoendoscopy for CRC surveillance in IBD colitis precluded a meaningful analysis of its impact on dysplasia detection and diagnosis since ≤10% of exams in our combined cohort were performed with chromoendoscopy. The reasons for the discrepancy in medical therapy

between the two groups are also unclear, but some possible explanations include a milder clinical course prompting less therapeutic intervention, possible liver test abnormalities in the PSC-IBD population raising clinicians' threshold for thiopurine and biologic use, and possible hesitancy of additional immunosuppression if PSC-IBD patients are post-liver transplant, particularly in the absence of clinical colitis symptoms. Lastly, we could not reliably assess the impact of PSC phenotype (e.g. small-duct PSC), ursodeoxycholic acid, liver tests abnormalities, or liver transplant/post-transplant immunosuppression on aCRN risk in the PSC-IBD population since this information was not universally available in the whole cohort.

The increased risk of CRC in patients with PSC and concomitant colonic IBD has firmly been established, although the underlying mechanism remains unclear[36].The nearly 3-fold higher rate of aCRN diagnosis following LGD detection, as well as the difference in location, morphology, and endoscopic conspicuousness of dysplasia in PSC-IBD compared to non-PSC IBD suggests nuances in the pathogenesis of neoplasia between these groups. Because the mechanisms underlying PSC as an independent risk factor for CRC in the setting of IBD colitis are unclear, the best strategy for CRC prevention in PSC-IBD remains frequent, attentive surveillance colonoscopy. Therefore, our findings suggest that while continued meticulous CRC surveillance with annual colonoscopy is indicated in the absence of dysplasia for PSC-IBD patients, and that the detection of LGD or higher-grade pathology should lead to a careful weighting of the pros and cons of more aggressive therapeutic management such as colectomy.

CONCLUSIONS

CONCLUSIONS

IBD are diseases with an increasing incidence worldwide, affecting young patients and with great impact in quality of life. In the past years, significant progress has been made in the management of IBD due to better knowledge about disease pathophysiology leading to the definition of new therapeutic targets and discovery of novel therapies. The association of IBD with PSC, despite being a rare event, carries a bad prognosis, due to higher likelihood of developing hepatobiliary cancer, colorectal cancer and/or greater risk of progressing to OLT or death[56]. PSC-IBD remains a disease where the few advances in the knowledge about pathophysiology have not yet translated into better treatment for patients and improved prognosis. In this thesis, we have tried to gain insight into some specific aspects of PSC-IBD natural history and pathophysiology:

1. Patients with PSC-IBD have a high risk of advanced colorectal neoplasia (aCRN) and higher rate of progression from low-grade to aCRN

Due to their high risk of developing colorectal dysplasia and cancer[76], patients with PSC-IBD need special colonic surveillance protocols. Therefore, all IBD societies recommend annual colonoscopy starting at the time of the PSC diagnosis, independently of the duration of the IBD. However, one important unanswered question in clinical practice remained the management of low-grade dysplasia (LGD) in these patients. This question is relevant, as in IBD alone, LGD progresses very rarely into more advanced forms of dysplasia (high-grade dysplasia or colorectal cancer), and many clinicians are reluctant to recommend a proctocolectomy in this situation, and rather continue an intensive surveillance protocol[309-311]. This approach has been gaining even more popularity due to better surveillance techniques (with the advent of high-definition scopes and chromoendoscopy) and better endoscopic management and ability to resect dysplastic mass lesions. However, not enough data was available in PSC-IBD.

The PhD candidate therefore, proposed to start a project looking into the natural history of LGD lesions in PSC-IBD. The project started at Mount Sinai Hospital, a tertiary center with a high-volume of PSC patients; during an investigator meeting the opportunity to collaborate with Dutch investigators who perform research in the area of dysplasia in IBD appeared, and a fruitful collaboration was started. We gathered a cohort of almost 300 PSC-IBD and 1600 IBD-alone patients, with a mean follow-up time of 4.8 years, with longitudinal data, multiple

surveillance colonoscopies and histopathology reports available. We confirmed the high risk for PSC-IBD patients to develop aCRN as compared to IBD alone, and we showed, unequivocally, that once patients with PSC-IBD have a diagnosis of LGD in their colon, there is a faster progression to aCRN (incidence rate of 1.3 per 100 patient-years as compared to 0.6 per 100 patient-years in IBD alone). Like in other cohorts, we also observed that colorectal neoplasia was more frequent in the right colon, albeit non-significantly. Importantly, and with an impact on the surveillance protocols we also showed that patients with PSC-IBD present more often invisible dysplasia, suggesting that in this subset of patients, the practice of performing random as opposed to targeted biopsies should probably not be abandoned. We thus believe our cohort has made a substantial contribution to the literature, with a direct impact on the care of patients.

2. Patients with PSC-IBD present lower expression of the primary bile acid receptor in their colon, as compared to IBD alone.

The reasons to why patients with PSC-IBD present with a unique phenotype characterized by extensive but mild colitis, and high risk for colorectal neoplasia (CRN) remains unknown. Since these patients have usually mild forms of colitis, other mechanisms besides inflammation must be at play to explain the increased risk of cancer.

BA homeostasis is tightly regulated by the activation of Farnesoid X Receptor (FXR), a nuclear bile acid receptor, expressed at high levels in liver and intestine[152], which plays an important role in entero-hepatic circulation of bile acids. It has been previously shown that intestinal inflammation decreases FXR cellular expression and that FXR knockout mice have an increased risk of colorectal cancer. We were able to show that FXR expression was diminished in dysplasia and cancer, with an inverse correlation within the neoplastic sequence (more severe degrees of dysplasia presented lower FXR expression), and was also globally down-regulated in PSC-IBD patients, independently of inflammation and more so in the right colon. This finding was unexpected, but led us to hypothesize that a lower expression of FXR in the right colon could expose colonocytes to higher levels of secondary bile acids or other toxic products, increasing carcinogenesis risk[312]. It is thus tempting to hypothesize that agonists of FXR could be an interesting approach for patients with PSC-IBD. As a matter of fact an FXR agonist, obeticolic acid, has been developed and approved for the treatment of primary biliary cholangitis[313], a related cholestatic liver disease.

Recently, positive results of the use of this agonist in PSC have been released by the Pharmaceutical company developing the drug[314].

3. PSC-IBD patients display a different mucosa-associated microbiome as compared to IBD patients alone

In the past years there has been increasing indirect evidence that luminal gut bacteria play a role in the pathogenesis of IBD[36], liver diseases[315], and colorectal cancer[316].

The first project that we developed at Mount Sinai, involved the collection of colonic biopsies from PSC-IBD patients, IBD alone patients and healthy volunteers. Considering the higher prevalence of neoplasia in the right colon in PSC-IBD patients, we specifically wanted to assess if there were any differences in the spatial distribution of the mucosa-associated microbiota between 3 different locations: terminal ileum, right and left colon. No significant site-specific differences in the microbial composition were found throughout the colon, suggesting that other mechanisms may be operating and interacting differently with the microbiota in the right as opposed to the left colon in patients with PSC-IBD. In this study, using bacterial 16S rRNA next-generation sequencing, we found, across all colonic locations, a consistent PSC-enrichment in *Blautia and Barnesiellaceae* genera and shifts in the *Clostridiales*, and less frequently under the *Bacteroidales* order. More specifically, around 86% of the relative changes in the microbiota occurred within the *Clostridiales* order, which is interesting as shifts in these taxa have also been observed in IBD and cirrhosis[206, 231]. It has been shown in the literature that the *Clostridiales* order, encompassing bacteria from *Lachnospiraceae* family, *Ruminococcaceae* family *and Blautia* genus, are able to perform 7α-dehydroxylation[208], an important step in converting primary to secondary bile acids in the intestine. Furthermore, in advanced cirrhosis, and in parallel, with a reduced level of fecalsecondary bile acids, a shift in the gut flora towards the enrichment of *Enterobacteriaceae* and the reduction of *Clostridiales* and *Bacteroidetes* has also been described[206]. Altogether, our findings point to a shift in the mucosa-associated microbiome in PSC-IBD, driven by changes in the BA pool; however, since we were not able to collect serum or stool bile acids we could only infer this conclusion from our ecological description and taxonomic findings.

4. PSC-IBD patients have a different fecal bile acid pool and microbiome and distinct microbiome-bile acid correlations

There is a close relationship between gut flora and bile acids (BA)[155], as BA have antimicrobial properties[317], and conversely, bile salt metabolism is a property of the gastrointestinal microflora. We collected paired serum and stool bile acids from patients with PSC-IBD and IBD alone and studied its correlations with the gut flora. All samples were collected from Portuguese patients, and then shipped to Mount Sinai for fecal microbiome study and analysis. Serum BA were studied at Prof. Cecilia Rodrigues´s lab, and the stool bile acid profiles in in Prof. Dominique Rainteau´s lab (Paris, France). We observed that patients with PSC-IBD displayed different fecal microbiota composition and stool BA profiles, as well as different correlations with the gut microbiota. Their stool bile acid pool was significantly reduced as compared to IBD alone. Although the individual stool bile acid components were not significantly different (possibly due to small sample size) between PSC-IBD and IBD, there was a non-significant increase in deoxycholic acid, a secondary bile acid with carcinogenic properties in vitro in PSC-IBD, and overall the fecal BA composition differed between the two groups. PSC-IBD patients presented an enrichment in bacteria belonging to the genera *Ruminococcus* and *Fusobacterium* as compared to IBD alone. At the OTU level, we observed a decrease in *Bacteroides*, *Clostridium, Bifidobacterium*, and *Lactobacillus* genus, taxa known to be involved in bile acid deconjugation[271]. Some of our findings are in consonance with recently published results on PSC microbiota also showing an increase of *Fusobacterium*[272], a bacterial taxon that has been linked with adenomas and colorectal cancer. We did observe an increase in the genus *Blautia* in IBD, which was in contrast with our findings in the mucosa-associated microbiome in our previous study. This may be explained by the reported differences between mucosa and stool microbiota[278], and by the fact that different patient populations with different geographical background were studied. Without any functional data, we may only speculate that under PSC condition, the BA changes may have dominant effects on defining the gut microbiota shifts, potentially towards a more pro-carcinogenic profile. Bacteria from both *Fusobacterium* and *Ruminococcus* genus, are known to be involved in oxidation, epimerization and desulfatation of bile acids[271]. Future studies should consider the functional properties of the gut microbiome, going beyond the mere genetic characterization allowed by 16S sequencing. Along this line, we have started a collaboration with Swiss investigators, looking at the activity of bile salt hydrolases in PSC-IBD and IBD

patients. In summary, despite all the advances provided by many investigators, including ourselves, in the characterization of the gut microbiome in the setting of PSC-IBD, it remains to be shown whether these changes are cause or consequence of altered bile acid pool, inflammatory milieu, or other. Most importantly, whether microbiome-based biomarkers could be used to screen for disease complications, or whether microbiome-based therapies could be used in PSC-IBD (as suggested by preliminary results) should also be explored.

It is clear that the gut-liver axis contributes to PSC pathogenesis and that further work unravelling this complex relationship could contribute to improve disease knowledge, potentially improving the lives of patients with this devastating condition.

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY MATERIAL

Supplementary tables

Supplementary Table 1

Primer ID	Sequence (5' to 3')
803R_b1	*AG***TCGACA**CTACCRGGGTATCTAATCC
803R_b2	*AG***GAACTC**CTACCRGGGTATCTAATCC
803R_b3	*AG***CTGTAG**CTACCRGGGTATCTAATCC
803R_b4	*AG***GACACG**CTACCRGGGTATCTAATCC
803R_b5	*AG***AGCGAG**CTACCRGGGTATCTAATCC
803R_b6	*AG***TCTCTA**CTACCRGGGTATCTAATCC
803R_b7	*AG***CGTGTC**CTACCRGGGTATCTAATCC
803R_b8	*AG***ATGCGT**CTACCRGGGTATCTAATCC
803R_b9	*AG***AACGCA**CTACCRGGGTATCTAATCC
803R_b10	*AG***ATTACC**CTACCRGGGTATCTAATCC
803R_b11	*AG***TGGTCA**CTACCRGGGTATCTAATCC
803R_b12	*AG***CCGTTT**CTACCRGGGTATCTAATCC
347F_b1	*AG***TCGACA**GGAGGCAGCAGTRRGGAAT
347F_b2	*AG***GAACTC**GGAGGCAGCAGTRRGGAAT
347F_b3	*AG***CTGTAG**GGAGGCAGCAGTRRGGAAT
347F_b4	*AG***GACACG**GGAGGCAGCAGTRRGGAAT
347F_b5	*AG***AGCGAG**GGAGGCAGCAGTRRGGAAT
347F_b6	*AG***TCTCTA**GGAGGCAGCAGTRRGGAAT
347F_b7	*AG***CGTGTC**GGAGGCAGCAGTRRGGAAT
347F_b8	*AG***ATGCGT**GGAGGCAGCAGTRRGGAAT
347F_b9	*AG***AACGCA**GGAGGCAGCAGTRRGGAAT
347F_b10	*AG***ATTACC**GGAGGCAGCAGTRRGGAAT
347F_b11	*AG***TGGTCA**GGAGGCAGCAGTRRGGAAT
347F_b12	*AG***CCGTTT**GGAGGCAGCAGTRRGGAAT

The sequences of 16S PCR primers targeted on 860bp amplicons for PacBio sequencing. The reversed primer sequences include 16 bp barcodes (lower case) at the 5'end

Forward Primer	
Blautia-404F	ATTGCACAATGGGGGAAACC
Reverse Primer	
Blautia-1263Rb1	gcgctctgtgtgcagcRTCATAAGGGGCATGATGAT
Blautia-1263Rb2	tcatgagtcgacactaRTCATAAGGGGCATGATGAT
Blautia-1263Rb3	tatctatcgtatacgcRTCATAAGGGGCATGATGAT
Blautia-1263Rb4	atcacactgcatctgaRTCATAAGGGGCATGATGAT
Blautia-1263Rb5	acgtacgctcgtcataRTCATAAGGGGCATGATGAT
Blautia-1263Rb6	tgtgagtcagtacgcgRTCATAAGGGGCATGATGAT
Blautia-1263Rb7	agagacacgatactcaRTCATAAGGGGCATGATGAT
Blautia-1263Rb8	ctgctagagtctacagRTCATAAGGGGCATGATGAT
Blautia-1263Rb9	agcactcgcgtcagtgRTCATAAGGGGCATGATGAT
Blautia-1263Rb10	tcatgcacgtctcgctRTCATAAGGGGCATGATGAT
Blautia-1263Rb11	agagcatctctgtactRTCATAAGGGGCATGATGAT
Blautia-1263Rb12	cgcatcgactacgctaRTCATAAGGGGCATGATGAT

Supplementary Table 1- 16S sequencing PCR primer sequences. The sequences of dual-barcoding 16S PCR primers targeted on 460bp amplicons for Miseq sequencing. The primer sequences include 2 bp linker (*italicized*) and 6 bp barcodes (**bold**) at the 5' end.

Supplementary Table 2

OUT ID	Ratio PSC/Control	Status	Taxonomy	P value
606927	43.18	Enr.	*Firmicutes; Clostridia; Clostridiales; Peptostreptococcaceae; [Clostridium]; difficile*	0.0364
189960	35.34	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;*	0.0197
4433417	33.83	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;*	0.0480
4447394	30.46	Enr.	*Actinobacteria; Actinobacteria; Actinomycetales; Propionibacteriaceae; Propionibacterium; acnes*	0.0317

188348	21.65	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0170
334340	21.09	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0069
4451907	18.37	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;*	0.0455
190653	18.29	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0451
574038	14.50	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0190
178977	13.44	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0162
180721	12.99	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0149
4372382	12.90	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0372
199501	11.63	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;*	0.0412
360660	11.39	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;*	0.0269
2317377	11.06	Enr.	*Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas;*	0.0375
335816	10.93	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0467
184174	10.67	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;*	0.0102
190058	9.95	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0405
2223978	9.75	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0303
186981	8.76	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;*	0.0247
189996	8.65	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0309
2177184	7.53	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0459
174672	7.14	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0267
182036	7.12	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0202
2119695	6.98	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0459
4423882	6.97	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus*	0.0499
808794	6.93	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0331
189530	6.90	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus];*	0.0249

563572	6.84	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0283
184114	6.66	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0225
187924	6.63	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0268
192461	6.53	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0410
4451899	6.08	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0275
4344861	6.04	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus*	0.0284
180878	6.00	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0145
2996838	5.99	Enr.	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0405
194758	5.79	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0077
3424669	5.61	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0451
174332	5.25	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;*	0.0395
190815	5.17	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;*	0.0106
4418787	5.13	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Roseburia;*	0.0014
195508	4.87	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; caccae*	0.0303
2724175	4.85	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0241
3090048	4.69	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0344
332210	4.67	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0474
4472158	4.63	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus*	0.0294
191361	4.41	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0343
182287	4.25	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0219
186772	4.21	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0368
177463	4.16	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0038
191913	4.12	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0291
198426	4.05	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0286
186168	3.86	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0459

199354	3.85	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;*	0.0205
194670	3.70	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;*	0.0338
4354582	3.65	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;*	0.0418
3702906	3.64	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus*	0.0497
191487	3.64	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;*	0.0480
199490	3.62	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0249
3715618	3.59	Enr.	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0369
189760	3.51	Enr.	*Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;*	0.0393
194008	3.48	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0259
183340	3.32	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;*	0.0288
3004856	3.30	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0365
194415	3.30	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0469
199694	3.29	Enr.	*Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium;*	0.0459
185281	3.21	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;*	0.0281
176197	3.15	Enr.	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0434
198751	3.15	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;*	0.0454
187572	3.09	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0282
174862	3.06	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0420
2762219	2.88	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0325
183662	2.75	Enr.	*Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;*	0.0483
191081	2.74	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0194
1860112	2.63	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0363
1602805	2.62	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;*	0.0103
187569	2.58	Enr.	*Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;*	0.0360
192462	0.21	Red	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0398
184036	0.15	Red	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0192

4358921	0.10	Red	*Firmicutes; Clostridia; Clostridiales; ; ;*	0.0490

Supplementary Table 2 - The fold changes and relative abundance of selected differential OTUs in PSC vs. healthy controls in the left colon biopsies. Table columns represent mean of the selected OTUs in each study group: controls, IBD and PSC. The ratio between mean OTUs in PSC and controls is represented with its corresponding taxonomy and *p value*. Enr: enriched; Red: reduced.

Supplementary figures

Supplementary Figure 1

After OTU picking, the fold changes and the relative abundance of selected differential OTUs in PSC vs. IBDs in the LC, RC and TI biopsies were compared. The bar plots show the fold changes of the relative abundance of each selected OTUs in PSC vs. non-PSC IBDs. The pink color indicates the enrichment in PSC. The green color indicates the reduction in PSC. The mean abundance in PSC, non-PSC IBD and healthy controls is shown on the right. red: healthy control; green: non-PSC IBD; blue: PSC. When compared with healthy controls, most of the PSC-associated shifts in the bacterial composition were observed in the *Clostridiales* and *Bacteroidetes* orders, with 86% in the former order. In agreement with our above findings, several PSC-enriched OTUs belonged to the *Blautia* genus and the *Barnesiellaceae* family. When compared to IBD, many OTUs were from the *Blautia* genus.

Supplementary Figure 2

After OTU picking, the fold changes and the relative abundance of selected differential OTUs in PSC vs. IBDs in the LC, RC and TI biopsies were compared. The bar plots show the fold changes of the relative abundance of each selected OTUs in PSC vs. non-PSC IBDs at each different location (terminal ileum, right and left colon). Similar enrichments and reductions at the PSC-associated OTUs selected from LC samples were observed in RC and TI locations.

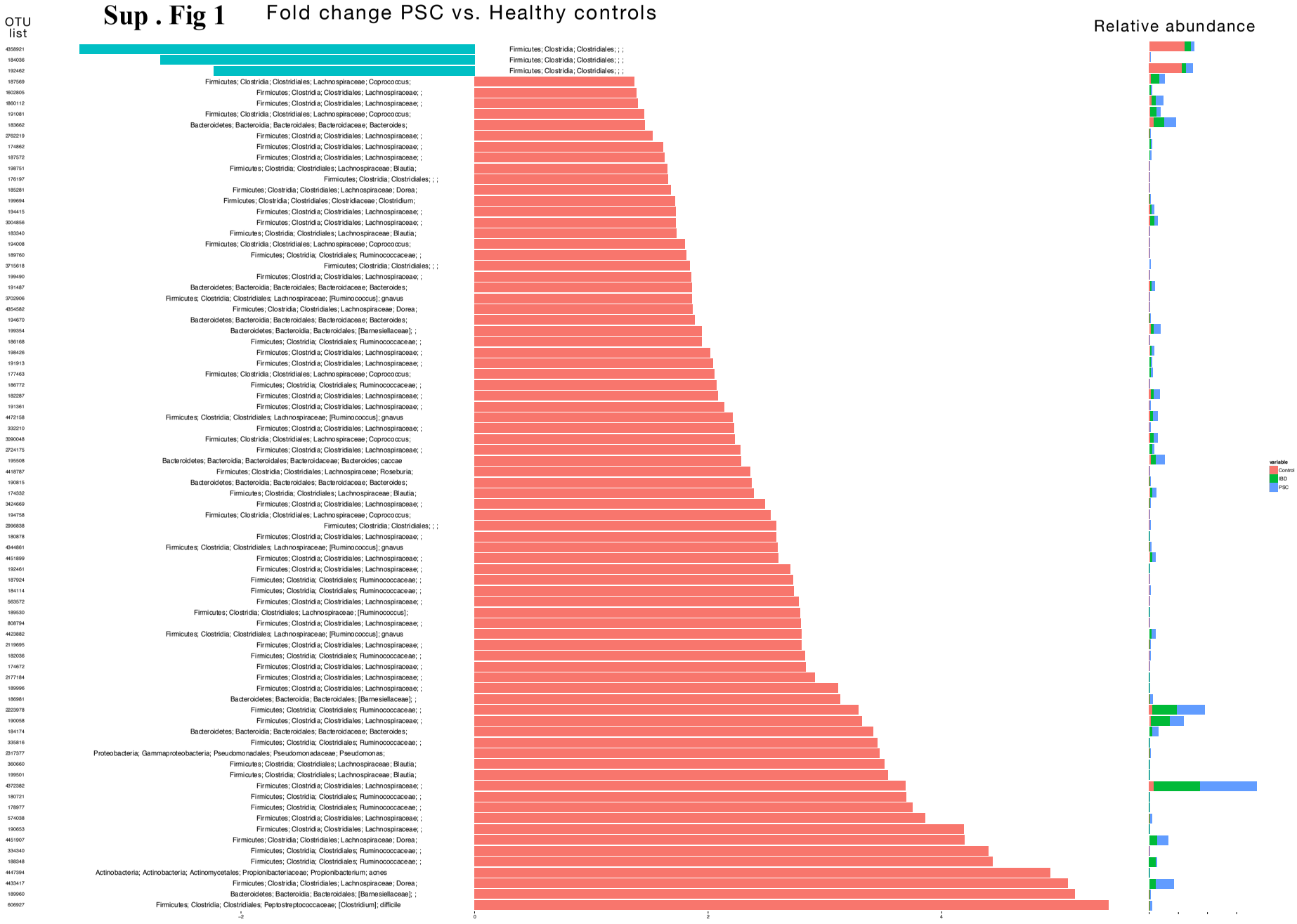
Supplementary Figure 3

The maximum likelihood phylogenetic tree within *Blautia* genus using UPGMA method was constructed. We combined 7 significantly PSC-associated OTUs and 15 reference sequences to construct the tree. The colored bar plots present the relative abundance of selected OTUs in PSC and non-PSC (combined healthy control and non-PSC IBDs) subjects. The table shows the number of differences (upper) and the percent identity (lower) calculated from pair-wise sequence alignment comparison. The color gradient from blue to red indicates the low to high sequence similarity.

Supplementary Figure 4

Results of the linear discriminant analysis. (A) all analytes (glycine and taurine BA conjugates, BA sulfates) were used. In the top panel, the *y* axis represents the linear discriminant coefficient. In the lower figure, in the y axis is represented the probability of being correctly classified as PSC-IBD using all stool BA. In both panels, the *x* axis each marker represents a patient. The green markers represent patients with PSC-IBD and in the pink markers patients with IBD. The circle represents patients that were correctly assigned to their disease group. The classification accuracy of the LDA using all bile acids was 100%. (B) The probability of being correctly assigned to the PSC-IBD group using only the top 4 most discriminative bile acids (GLCA, TLCA, TUDCA, and TLCA3S) is illustrated; the table shows the performance of the model to classify correctly PSC-IBD.

Sup . Fig 1


Fold change PSC vs. Healthy controls
OTU list
Relative abundance
4358921 Firmicutes; Clostridia; Clostridiales; ; ;
184036 Firmicutes; Clostridia; Clostridiales; ; ;
192462 Firmicutes; Clostridia; Clostridiales; ; ;
187569 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
1602805 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
1860112 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
191081 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
183662 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
2762219 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
174862 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
187572 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
198751 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
176197 Firmicutes; Clostridia; Clostridiales; ; ;
185281 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;
199694 Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium;
194415 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
3004856 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
183340 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
194008 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
189760 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
3715618 Firmicutes; Clostridia; Clostridiales; ; ;
199490 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
191487 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
3702906 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus
4354582 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;
194670 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
199354 Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;
186168 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
198426 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
191913 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
177463 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
186772 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
182287 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
191361 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
4472158 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus
332210 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
3090048 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
2724175 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
195508 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides; caccae
4418787 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Roseburia;
190815 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
174332 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
3424669 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
194758 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
2996838 Firmicutes; Clostridia; Clostridiales; ; ;
180878 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
4344861 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus]; gnavus
4451899 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
192461 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
187924 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
184114 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
563572 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
189530 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; [Ruminococcus];
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2119695 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
182036 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
174672 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
2177184 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
189996 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
186981 Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;
2223978 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
190058 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
184174 Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides;
335816 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
2317377 Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas;
360660 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
199501 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
4372382 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
180721 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
178977 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
574038 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
190653 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
4451907 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;
334340 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
188348 Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
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4433417 Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Dorea;
189960 Bacteroidetes; Bacteroidia; Bacteroidales; [Barnesiellaceae]; ;
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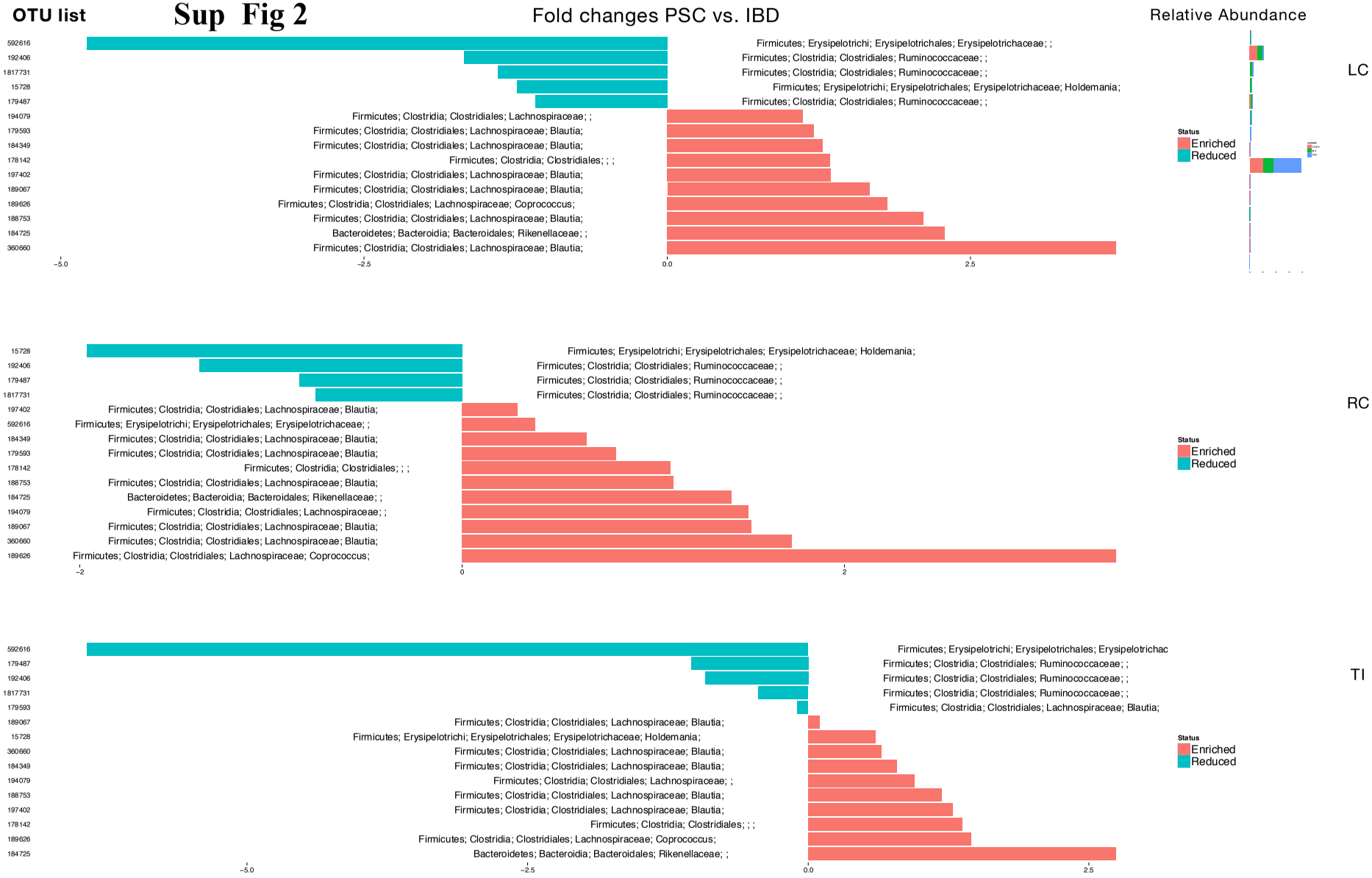
variable
Control
IBD
PSC

Sup Fig 2


OTU list
Fold changes PSC vs. IBD
Relative Abundance
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1817731
15728
179487
194079
179593
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197402
189067
189626
188753
184725
360660
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; ;
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
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Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Holdemania;
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; ;
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; ;
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Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; ;
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Blautia;
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2.5
Status
Enriched
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LC
15728
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178142
188753
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194079
189067
360660
189626
Firmicutes; Erysipelotrichi; Erysipelotrichales; Erysipelotrichaceae; Holdemania;
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Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
−2
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Status
Enriched
Reduced
RC
592616
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178142
189626
184725
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Firmicutes; Clostridia; Clostridiales; ; ;
Firmicutes; Clostridia; Clostridiales; Lachnospiraceae; Coprococcus;
Bacteroidetes; Bacteroidia; Bacteroidales; Rikenellaceae; ;
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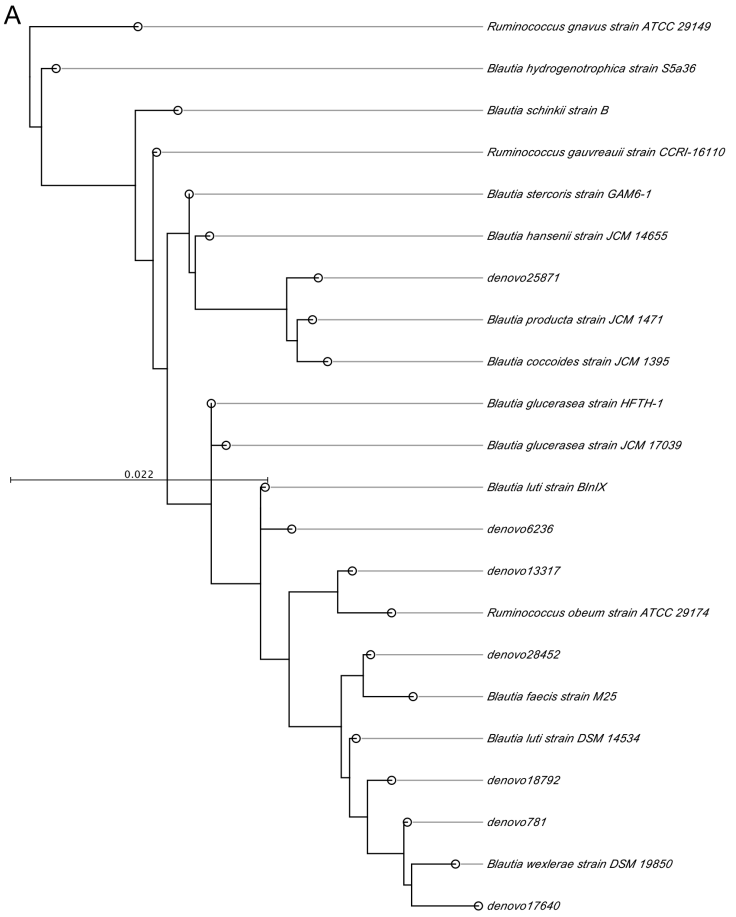
−2.5
0.0
2.5
Status
Enriched
Reduced
TI

Sup Fig. 3

A



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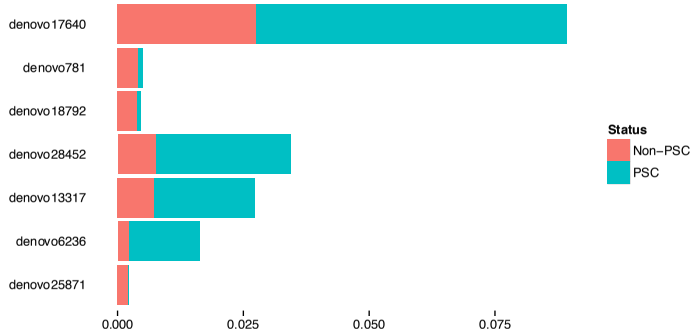

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denovo18792	6	92.29	92.29	93.24	93.60	94.42		74	71	71	74	75	76	83	84	84	85	85	79	81	49	90	90
denovo13317	7	96.49	96.49	96.73	96.13	96.25	91.22		5	35	31	32	50	34	42	42	44	37	31	37	75	49	56
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denovo28452	10	96.61	96.50	95.89	95.29	96.49	91.22	96.25	96.25	95.41		1	49	45	39	39	41	41	35	45	75	52	64
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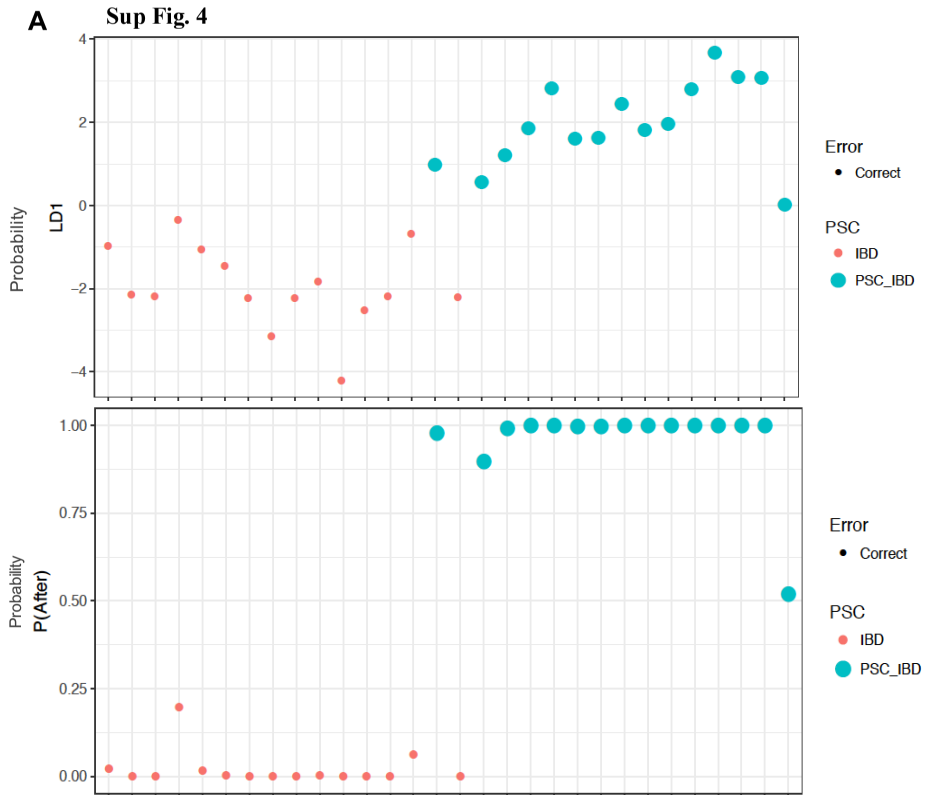
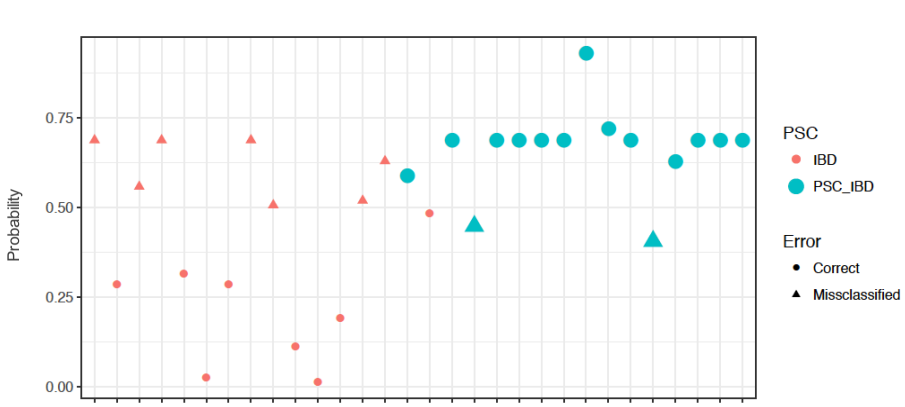
gi|343201234|ref|NR_041960.1|:247–1075	13	94.71	94.59	94.46	94.22	94.71	90.19	95.91	95.79	94.83	94.59	94.47	93.76		54	54	55	51	41	35	85	64	62
gi|631252072|ref|NR_113270.1|:373–1199	14	96.01	96.01	94.32	94.69	93.95	90.04	94.92	95.16	96.37	95.28	95.16	94.20	93.51		1	9	25	29	48	82	39	54
gi|559795114|ref|NR_104700.1|:373–1199	15	96.01	96.01	94.32	94.69	93.95	90.04	94.92	95.16	96.37	95.28	95.16	94.20	93.51	99.88		9	24	29	48	82	39	53
denovo25871	16	95.77	95.65	93.95	94.32	93.83	89.92	94.68	94.68	96.25	95.04	94.92	93.84	93.39	98.91	98.91		27	30	49	83	43	57
gi|559795101|ref|NR_104687.1|:373–1198	17	95.28	95.29	95.28	95.05	94.44	89.92	95.53	95.89	96.25	95.04	94.92	93.12	93.87	96.98	97.10	96.74		26	50	84	48	55
gi|645320731|ref|NR_117867.1|:325–1151	18	96.01	95.89	95.16	94.57	95.65	90.63	96.25	96.13	96.13	95.77	95.65	94.20	95.07	96.49	96.49	96.37	96.86		46	80	45	60
denovo6236	19	95.17	95.06	94.45	94.70	94.46	90.40	95.54	95.54	94.70	94.58	94.46	93.26	95.80	94.22	94.22	94.10	93.98	94.46		76	56	61
denovo781	20	92.60	92.60	93.91	93.92	93.92	94.12	91.05	91.41	91.54	91.06	90.94	91.07	89.92	90.23	90.23	90.11	89.99	90.46	90.95		86	88
gi|343205824|ref|NR_044265.1|:355–1181	21	94.33	94.22	93.85	93.73	93.85	89.35	94.09	94.21	95.78	93.73	93.61	93.61	92.33	95.30	95.30	94.81	94.21	94.57	93.27	89.76		48
gi|310974936|ref|NR_036800.1|:351–1177	22	93.96	94.08	93.24	93.61	93.12	89.34	93.24	93.60	94.81	92.28	92.16	92.89	92.56	93.49	93.61	93.12	93.37	92.76	92.66	89.50	94.22	

Sup Fig. 4





Sensitivity	0.87
Specificity	0.53
Pos Pred Value	0.65
Neg Pred Value	0.8
Balanced Accuracy	0.7

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ADDENDUM

FARNESOID X RECEPTOR EXPRESSION IS DECREASED IN COLONIC MUCOSA OF PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS AND COLITIS-ASSOCIATED NEOPLASIA

Farnesoid X Receptor Expression Is Decreased in Colonic Mucosa of Patients with Primary Sclerosing Cholangitis and Colitis-associated Neoplasia

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Background: The expression and distribution of farnesoid X receptor (FXR) in colitis and colitis-associated neoplasia (CAN) is unknown. We investigated FXR expression in neoplastic and nonneoplastic tissue from ulcerative colitis (UC) patients, with or without primary sclerosing cholangitis (PSC), as well as the role of DNA methylation in FXR expression in colorectal cancer (CRC) cell lines.

Methods: Samples from the right (RC) and left (LC) colon of patients with UC, with and without PSC, and with or without CAN, were stained by immunohistochemistry and scored semiquantitatively for nuclear FXR expression. FXR expression was analyzed by western blot and polymerase chain reaction (PCR) in nine different CRC cell lines before and after demethylation with 5-azacytidine.

Results: In nondysplastic samples, FXR expression demonstrated a diminishing expression from proximal to distal colon (strong FXR expression: 39% RC samples vs. 14% LC samples; $P = 0.007$). With moderate-to-severe inflammation, FXR expression was almost always absent or weak in both UC and PSC-UC, regardless of location. With quiescent/mild inflammation, 56% of UC samples in the RC retained strong FXR expression versus 24% of PSC-UC samples ($P = 0.017$). FXR was absent in 72% of the neoplastic samples, with an inverse association with the grade of dysplasia. FXR expression was absent in all CRC cell lines, in some cases due to DNA methylation.

Conclusions: FXR expression is inversely correlated with neoplastic progression and severity of inflammation in UC. Patients with PSC-UC have diminished FXR expression in the proximal colon compared to UC patients. This finding could contribute to the higher risk of proximal neoplasia in PSC patients.

(Inflamm Bowel Dis 2013;19:275–282)

Key Words: primary sclerosing cholangitis, colorectal neoplasia, farnesoid X receptor

Patients with inflammatory bowel disease (IBD) are at increased risk for developing colitis-associated neoplasia (CRN).[1] Risk factors for developing CRN in the setting of IBD include longer disease duration, more extensive inflammation of the colonic mucosa, family history of sporadic colon cancer, endoscopically or histologically active inflammation, and associated primary sclerosing cholangitis (PSC).[2] Of all these risk factors, PSC confers the highest risk of developing colorectal neoplasia, ≈5-fold in some studies.[3] Chronic inflammation is considered a very strong promoter of colon carcinogenesis.[2,4] Intriguingly, despite their very high colorectal cancer (CRC) risk, patients with PSC-IBD typically have

Received for publication May 16, 2012; Accepted May 31, 2012.

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Joana Torres received a Travel Grant from International Organization for the study of Inflammatory Bowel Disease (IOIBD) and a grant from the Burrill B. Crohn Research Foundation. This work was supported in part by the Gastric Cancer and Peptic Ulcer Disease Foundation and the Chemotherapy Foundation.

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DOI 10.1097/MIB.0b013e318286ff2e

Published online 23 January 2013.

colitis that is very mild, with little or no symptoms and minimal changes on colonoscopy. This high risk of CRC in PSC-IBD patients in the setting of little inflammation poses an intriguing paradox regarding inflammation-associated colonic carcinogenesis.

Cancer risk may vary depending on the location in the colon in patients with IBD. We recently demonstrated that dysplasia and cancer occur more often in the left colon (LC) of patients with extensive UC.[5] Moreover, progression from low-grade dysplasia (LGD) to high-grade dysplasia (HGD) or CRC in UC patients is more common in the LC compared to the right colon (RC).[6] In contrast, patients with PSC in the setting of UC have been reported to have higher neoplasia risk in the proximal colon, for reasons that are yet to be explained.[7] One theory is that altered bile acid composition and/or concentration in PSC patients, which may be procarcinogenic, affect the proximal colon more so than the distal colon.[8]

Bile acids have been implicated as causative factors in colorectal carcinogenesis.[9] Patients with UC and dysplasia or carcinoma have been shown to have higher concentrations of bile acids in feces as compared to UC patients without neoplasia.[10] Additionally, exposure of colonocytes to a higher load of secondary bile acids has been proposed as an explanation for the increased risk of proximal CRN observed in the subset of patients with PSC.[8]

Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily, highly expressed in the liver and terminal ileum, where it functions as the primary sensor for bile acids and regulates cholesterol, fatty acid, and glucose metabolism.[11] Recently, FXR has also been shown to have a role in maintaining the integrity of the epithelial barrier and in innate immunity.[12,13] In the colon, FXR expression has been inversely correlated with both inflammation and carcinogenesis. FXR- knockout mice are more susceptible to chronic intestinal inflammation and, conversely, FXR activation protects against intestinal inflammation in mice, possibly by repressing nuclear factor kappa-B and by counterregulating inflammatory cytokine expression in immune cells.[14,15] FXR mRNA expression seems to be decreased in colon biopsies from macroscopically inflamed mucosa of patients with Crohn's disease (CD) but maintained in those with a normal appearance at colonoscopy.[13]

With respect to neoplasia, FXR seems to function as a tumor suppressor. For example, FXR mRNA expression is decreased in sporadic adenomatous polyps, an effect that is even more pronounced in colonic adenocarcinoma.[16] Furthermore, $APC^{-/+}$ mice developed more intestinal neoplasms when they were crossed with $FXR^{-/-}$ mice.[17]

In the present study we evaluated FXR expression by immunohistochemistry in colonic mucosa of patients with UC, with or without PSC, and correlated this with location in the colon, degree of inflammation, and presence or absence of CRN. We also measured FXR mRNA expression in CRC cell lines and explored the influence of DNA methylation in FXR expression in these cell lines.

MATERIALS AND METHODS

Case Selection

Patients were identified using the Mount Sinai GI Pathology and UC-Surveillance databases described in previous studies.[18] Besides demographic information, clinical information such as extraintestinal manifestations and pathologic findings from colonoscopies and operations, including anatomic extent of disease at diagnosis, presence and grade of any dysplasia, and presence and severity of inflammation at each biopsied segment of colon were recorded. Additionally, for the measurement of FXR expression in colitis-associated neoplasms, we analyzed samples in two tissue microarrays (TMAs) containing samples of CRN from IBD patients (CRC, low- and high-grade dysplasia).

Patients with extensive UC at diagnosis, with or without PSC, were identified and their pathology blocks retrieved. The presence or absence of PSC was based on the results of serum biochemical tests and cholangiography and/or hepatic biopsy. Patients with CD and indeterminate IBD were excluded. From each patient, whenever available, samples from the RC and LC were recovered. An attempt was made to have samples representative of different degrees of histological inflammation.

In all, 112 patients (84 males, median age 51 years) were included in this study. All patients had extensive UC at diagnosis (defined by greatest microscopic extent proximal to the splenic flexure). Median disease duration for the patients in whom that information was available was 20 ± 13 years (range 1–60 years). Twenty-one patients had no neoplasia in the colon, whereas the others had neoplasia somewhere in the colon. There were 24 patients with concomitant PSC. From all these patients, 231 colonic samples (biopsy specimens, surgical samples, and tissue microarrays) were obtained: 155 from UC patients (62 from the RC) and 76 from PSC-UC (39 from the RC).

Histologic Grading of Inflammation and Neoplasia

For each nondysplastic sample, the severity of histologic inflammation was taken from the pathology report. These specimens had been scored according to the histologic activity index (HAI) that we previously validated and described.[18] Briefly, the degree of inflammation for each biopsy site was scored as: absent or quiescent (0); mildly active (1); moderately active (2); or severely active (3). Dysplasia was diagnosed initially by one of the authors (N.H.) using the criteria of the IBD Dysplasia Morphology Study Group[19] and confirmed as a part of this study by a second GI pathologist (A.C.I.).

Immunohistochemistry

Immunohistochemistry for FXR was performed manually on samples and tissue microarrays, using a mouse antihuman FXR monoclonal antibody (Perseus Proteomics, Tokyo, Japan). This antibody specifically recognizes human FXR and crossreacts with mouse and rat FXR. From the formalin-fixed, paraffin-embedded tissue blocks of biopsies and colectomy specimens, sequential sections were cut at 4-μm thickness and mounted on adhesive slides. Slides were deparaffinized in xylene and subsequently washed in graded ethanol (100%, followed by 95%) and rehydrated in distilled water. For antigen retrieval, sections were incubated in a microwave for 30 minutes using a 0.1% sodium citrate buffer and subsequently washed in phosphate-buffered saline (PBS) at room temperature. Endogenous peroxidase activity was blocked by incubating the slides with 3% H_2O_2 for 10 minutes and then rinsed three times with PBS. Sections were incubated for 60 minutes at room temperature in 2% bovine serum albumin (BSA) to avoid nonspecific signal, and then overnight at 4°C with the primary anti-FXR antibody. Subsequently, slides were rinsed three times in PBS and treated for 30 minutes at room temperature with a polyclonal antimouse secondary antibody (EnVision+ System-HRP Labeled Polymer Anti-mouse; Dako, Glostrup, Denmark), and again washed three times with PBS. Slides were then incubated with diaminobenzidine (DAB) using the peroxidase substrate DAB kit (Vector Laboratories, Burlingame, CA). After cleansing with water, slides were counterstained with Harris modified hematoxylin solution for 50 seconds, dipped in ethanol and in ammonia water, and rinsed in tap water in between. Finally, sections were dehydrated in 95% and 100% alcohol consecutively, washed with xylene, and mounted with VectaMount (Vector Laboratories). At least one section with normal small intestinal mucosa was included for each run as a positive control.

Evaluation of Immunohistochemistry

FXR nuclear expression was scored by two independent, experienced observers (S.I. and A.C.I.) who were blinded to the clinicopathological information. FXR nuclear staining intensity was scored as absent (0), weak (+), and strong (++). There was ≈95% concordance between the two observers. Differences were resolved by consensus evaluation of the slides.

FXR Gene Demethylation Experiments

Cell Culture

Human liver cell line HepG-2 cells were cultured in RPMI-1640 medium (Invitrogen, La Jolla, CA) with 10% fetal bovine serum (FBS) cell at 37°C in 5% CO_2 atmosphere. Human colorectal cancer cell lines (Caco-2, HCT-116, HT-2, and SW-480 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% FBS Cell at 37°C in 5% CO_2 atmosphere. HepG-2, Caco-2, HCT-116, HT-2, and SW-480 cells were seeded at a concentration 1×10^4 cells/mL in 6-well cell culture plate, 2 mL/well, and the following day, were treated with 0, 1, and 10 μM 5-aza-2′-deooxycytidine (5-aza) (Sigma-Aldrich, St. Louis, MO) in fresh culture medium. 5-aza was removed by replacing the medium 24 hours later. The cells were harvested 4 days after removal of 5-aza for RNA extraction.

Quantitative Real-time PCR Analysis of FXR mRNA Expression

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Chatsworth, CA). One μg total RNA was used for cDNA synthesis in RNA to DNA EcoDry Premix (Double Primed) (ClonTech, Palo Alto, CA). FXR mRNA levels were evaluated by SYBE Green assay using advantage qPCR premix (ClonTech) and primers: 5′-GCCTGTCTCCTGGGTCGCCT-3′ (forward) and 5′-TCCCCAT-CACTGCACGTCCCA-3′ located in exon 11. The mRNA level of the target genes was normalized to GAPDH mRNA. Primers of GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCAC-CACCCTGTTGCTGTA-3′. All samples were analyzed in triplicate.

Statistical Analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS for Mac, v. 19.0, Chicago, IL). When appropriate, Fisher's exact test, Student's *t*-test, and χ^2 tests were used for comparison between groups. Statistical significance was set at $P < 0.05$.

Ethical Considerations

This study was approved by the Mount Sinai School of Medicine Institutional Review Board.

RESULTS

FXR Expression in Nonneoplastic Mucosa

There were 152 samples without dysplasia from 62 patients: 66 from 22 PSC-UC patients, and 86 from 40 UC patients. Among the nondysplastic samples, 70 came from 21 patients without neoplasia, while 82 came from patients with neoplasia elsewhere in the colon. Seventy-two samples were from the RC (mostly ascending colon and proximal transverse) and 80 from the LC (mostly sigmoid colon).

We observed that FXR expression was higher in the RC than the LC. Overall, 32% of the samples from the RC demonstrated strong expression, compared to only 14% in the LC ($P = 0.011$). When stratified by disease type, this difference remained statistically significant only for UC samples: 39.5% of the samples from the RC versus 16.7% had strong FXR expression ($P = 0.017$). In PSC-UC samples, while there was also a trend toward stronger FXR expression in the proximal colon (strong FXR expression in 23.5% of samples from RC vs. 9.4% from LC), this did not reach significance ($P = 0.11$). Thus, FXR expression seems to be weaker in the

TABLE 1. Relationship Between Degree of FXR Expression and the Degree of Inflammation According to Colonic Location and Disease Type

Inflammation	Location	Disease type (n)	Absent or Weak FXR Expression n (%)	Strong FXR Expression n (%)	P value
Quiescent-mild	RC	PSC-UC (29)	22 (75.9)	7 (24.1)	0.017
		UC (25)	11 (44)	14 (56)	
	LC	PSC-UC (29)	26 (89.7)	3 (10.3)	0.52
		UC (30)	26 (86.7)	4 (13.3)	
Moderate-severe	RC	PSC-UC (5)	4 (80)	1 (20)	0.49
		UC (13)	12 (92.3)	1 (7.7)	
	LC	PSC-UC (3)	3 (100)	0 (0)	0.51
		UC (18)	14 (77.8)	4 (22.2)	

LC of patients with UC and PSC-UC. FXR expression did not differ according to location or disease type in samples from patients without neoplasia compared to those with neoplasia somewhere in the colon (data not shown).

When we compared FXR expression to the degree of inflammation, as expected, samples with the highest degrees of inflammation (moderate or severe inflammation) demonstrated FXR expression that was almost always absent or weak in both UC and PSC-UC, regardless of colonic location (Table 1). Very few of the highly inflamed samples demonstrated strong FXR expression. With quiescent or mild inflammation, FXR expression was almost always absent or weak in the LC (87% in UC, 90% in PSC-UC), reflecting the normal distal decrease in FXR expression (Fig. 1E,F). In samples from the RC of UC patients, FXR expression decreased with higher degrees of inflammation: 56% of specimens with quiescent/mild inflammation had strong FXR expression, compared to 7.7% (1/13) with moderate/severe inflammation ($P = 0.005$). Unexpectedly, the same pattern of FXR expression was not observed in the RC of PSC-UC patients: 24% of the RC samples displaying quiescent/mild inflammation had strong FXR expression compared to 20% of samples with moderate/severe inflammation (Fig. 1B,C). Indeed, the main difference between PSC-UC and UC was the finding that in the RC with quiescent or mild inflammation 56% of UC samples retained strong FXR expression, compared to only 24% of PSC-UC samples ($P = 0.017$) (Table 1; Fig. 1).

Similar to previous observations in normal colon,[20] we observed that FXR immunoreactivity presented a gradient of expression throughout the crypts, which was especially evident in the RC. Thus, FXR expression was stronger at the crypt surface and gradually decreased toward the base of the crypts, where it was often absent (see Fig. 1B).

FXR Expression in Neoplastic Mucosa

From 9 PSC-UC and 62 UC patients, 79 samples of CRN (19 colonoscopic biopsies and 60 samples from the TMAs) were analyzed. There were 52 CRC (15 well differentiated; 14 moderately differentiated; 23 poorly differentiated), six high-grade dysplasia (HGD) and 21 low-grade dysplasia (LGD).

Overall, FXR expression was absent in most (57/79; 72.2%) of the dysplastic and CRC samples. Among the samples maintaining any FXR expression, there was an inverse correlation with the degree of neoplasia (Fig. 2). FXR was expressed in LGD (13/21; 61.9%), HGD (3/6; 50%), and CRC (6/52; 11.5%), although the difference was significant only between dysplastic samples versus cancer (Table 2).

No significant differences were observed according to colonic location of the neoplasm, or between UC and PSC-UC patients (data not shown). In the CRC samples that retained some weak expression, no differences were found according to cancer differentiation.

In Vitro Studies with Colon Carcinoma Cell Lines

Compared to the HepG2 cell line, FXR expression at the mRNA level was very low, and was undetectable by western blot in all nine CRC cell lines. In HCT-116 and SW480 cells, 5-aza treatment resulted in dose-dependent increases in FXR mRNA expression. There were no significant changes in FXR mRNA expression in Caco-2, SW480, and HepG2 cells after treatment with 5-aza (Fig. 3).

DISCUSSION

The involvement of the FXR nuclear bile acid receptor in both intestinal inflammation and carcinogenesis makes it an interesting target to study in colitis and in colitis-associated neoplasia. Furthermore, being the major regulator of the enterohepatic circulation of bile acids, we specifically wanted to analyze the influence of concomitant PSC in FXR's colonic expression.

A recent study showed that there was no difference in ileal and ascending colon FXR mRNA expression between controls, CD, and UC patients in remission.[21] However, the distribution of FXR throughout the colon of patients with UC, or the regional distribution and relation to inflammation in PSC-UC patients, has not previously been reported. In UC patients, we observed a distal

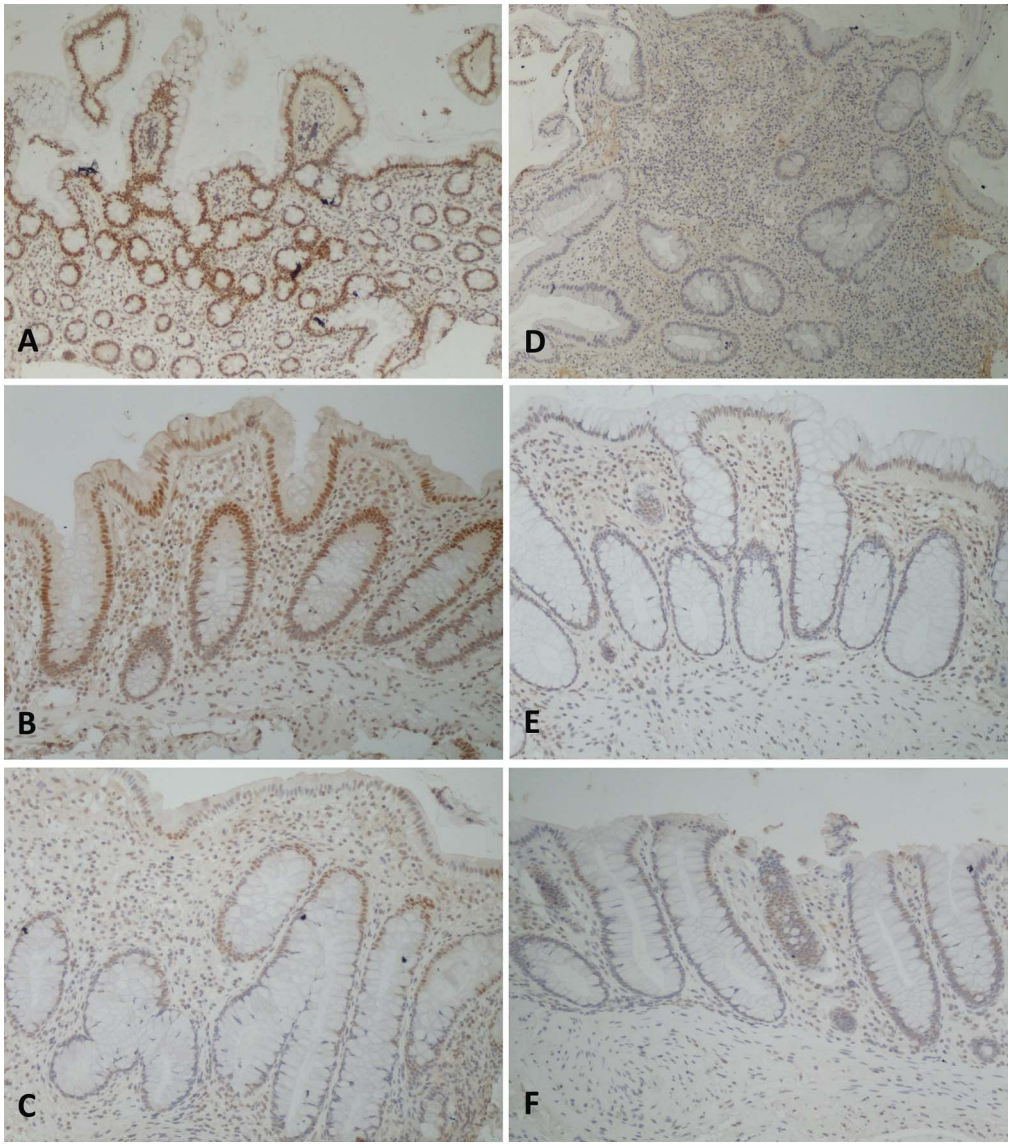

FIGURE 1. FXR immunohistochemical staining of nonneoplastic mucosa. (A) Normal terminal ileum from patient with UC: there is strong nuclear staining in villi with gradual decrease towards the crypts (10×). (B) RC biopsy of a patient with UC and quiescent inflammation; a strong nuclear positivity at the surface and upper part of the colonic glands with a gradual loss of expression in the crypts is seen (20×). (C) RC biopsy of patient with PSC and quiescent inflammation displaying diminished FXR nuclear expression (20×). (D) RC of a patient with UC and moderate inflammation: decreased FXR nuclear expression compared to uninflamed mucosa in (B) (20×). (E) Uninflamed LC in a patient with UC lacks strong FXR staining (20×). (F) Uninflamed LC of patient with PSC-UC displays only minimal or no FXR nuclear staining (20×).

decrease in FXR expression, with a stronger expression in the RC samples as compared to the LC samples. This pattern of distribution has been described in normal subjects, and proposed to occur in parallel with the proximal-distal gradient of bile acid flow along the colon.[20] However, when we stratified by disease type we observed decreased FXR expression in samples from the RC of PSC-UC patients.

Since FXR has been shown to be downregulated in the presence of intestinal inflammation, we also analyzed the results according to the degree of histologically active inflammation. Perhaps not unexpectedly, we observed that with more severe inflammation, FXR expression was usually absent or weak, independent of location or disease type. With quiescent/mild inflammation, FXR expression was also typically weak/absent in both UC and PSC-UC patients if the tissue sample was from the LC, reflecting the normal distal decrease in FXR expression. Interestingly, with little or no inflammation, the RC of UC patients retained the normal pattern of rather high expression of FXR, but the RC of PSC-UC patients did not. Thus, PSC-UC patients appear to lose FXR expression in the RC even without much inflammation.

There are data implicating FXR as a tumor suppressor gene.[22] FXR is decreased in human sporadic CRC with an inverse

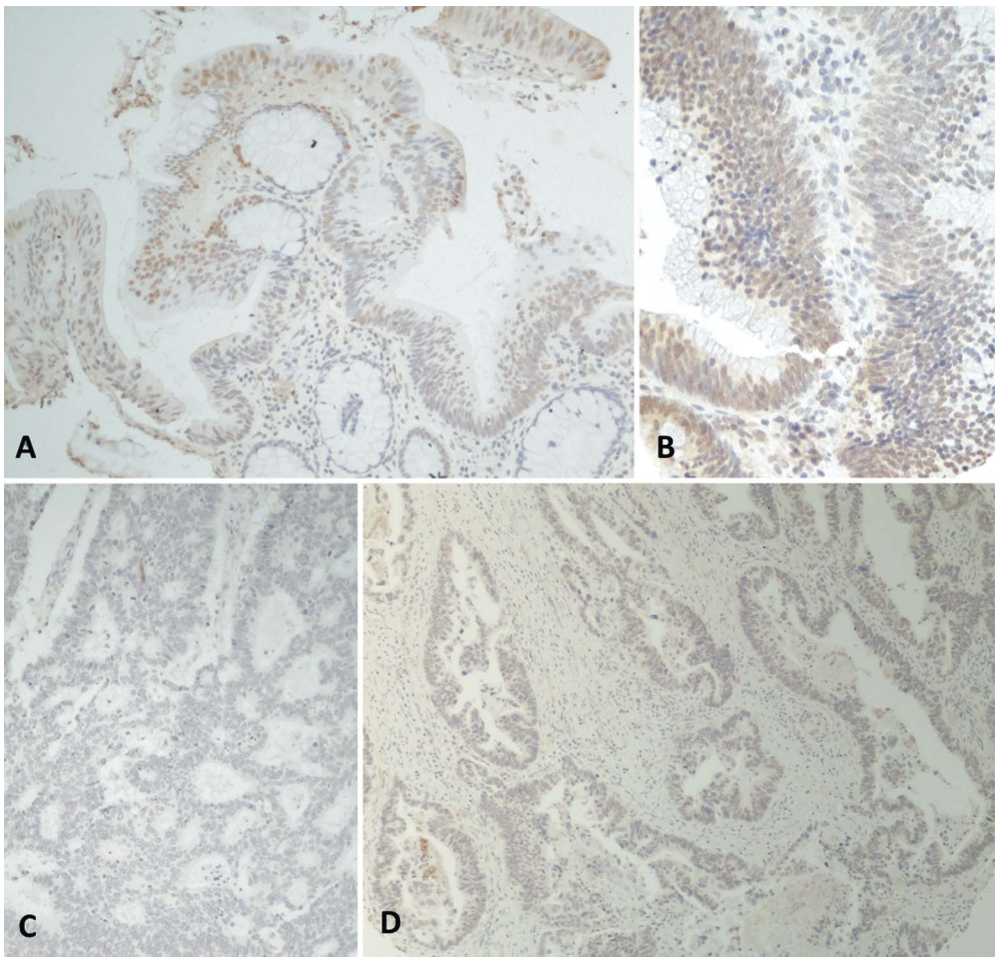

FIGURE 2. Examples of colitis-associated neoplasia. (A) LGD showing retained FXR nuclear expression (20×). (B) High-power image of a focus of LGD highlights nuclear FXR positivity (40×). (C) HGD: loss of FXR expression (20×). (D) Invasive adenocarcinoma: loss of FXR expression (20×).

correlation between the degree of expression and tumor stage.[16,20] Furthermore, $FXR^{-/-}$ mice have been shown to have increased intestinal carcinogenesis, increased cell proliferation via promotion of Wnt signaling, and upregulation in the expression of genes involved in cell cycle progression and inflammation, such as cyclin D1 and interleukin-6.[17] The expression of FXR in the different stages of colitis-associated neoplasia had not yet been described. We herein observed that there was an inverse correlation within the neoplastic sequence in colitis-associated carcinogenesis, with complete loss of FXR expression in 38%, 50%, and 88.5% of LGD, HGD, and colitis-associated cancers, respectively. FXR expression in the nonneoplastic adjacent mucosa did not present a similar pattern of expression (data not shown), and therefore it is likely that FXR downregulation occurs during neoplastic transformation from LGD, to HGD, and finally adenocarcinoma, rather than representing a field defect.

TABLE 2. Relationship Between FXR Expression and Degree of Colitis-associated Neoplasia

				P value	
CRN	Absent FXR expression *n* (%)	Low FXR expression *n* (%)	Strong FXR expression *n* (%)	LGD	HGD
LGD (n=21)	8 (38.1%)	13 (61.9%)	0	ns	
HGD (n=6)	3 (50%)	2 (33.3%)	1 (16.7%)		
CRC (n=52)	46 (88.5%)	6 (11.5%)	0	< 0.001	0.003

ns, not significant.

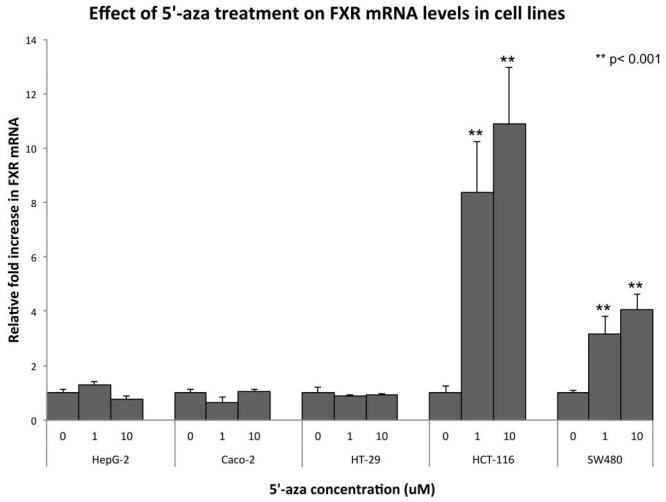

FIGURE 3. Effect of 5-aza (0, 1, 10 μM) treatment on FXR mRNA levels in CRC cell lines (μM - micromol).

We additionally sought to explore the potential role of DNA methylation as a molecular mechanism of downregulation of FXR mRNA expression in colorectal cancer cell lines. DNA methylation is a common mechanism occurring in the development and progression of sporadic CRC, which may also have a role in colitis-associated neoplasia.[23] Putative CpG islands within the promoter and the fourth exon of the human FXR gene have previously been described.[24] We observed that while in Caco-2, HT-29, and HepG2 cell lines there where no changes in FXR mRNA expression after 5-aza treatment, in HCT-116 and SW480 cells, 5-aza treatment resulted in dose-dependent increases in FXR mRNA expression. This suggests that loss of expression of FXR in colonic cells may be regulated, at least in part, by DNA methylation.

Bearing in mind the role of FXR in colorectal carcinogenesis, it is tempting to suggest that FXR downregulation observed in PSC-UC samples could explain, in part, the high risk of right-sided CRN in these patients, even in the presence of quiescent to mild inflammation. There are data suggesting that during cholestatic liver disease, such as PSC, intestinal bile acid absorption is reduced which could lead to a relative increase of bile acid in the proximal colon, in turn producing heightened conversion of primary bile acids into secondary, more carcinogenic, bile acids.[25,26] Downregulation of FXR could therefore expose colonocytes to high levels of secondary bile acids or other toxic products, increasing carcinogenesis risk.

Our study has some limitations. Although a large number of samples were studied overall, the difference of FXR expression in the RC of PSC-UC patients was based on a somewhat small sample size. These findings should be confirmed in other studies. In addition, as a descriptive, retrospective immunohistochemical study on archival tissue, our study does not allow us to draw any mechanistic conclusions, such as whether low FXR expression results from FXR downregulation or from posttranscriptional events modulating FXR expression. Further research is required to elucidate the underlying mechanisms for FXR downregulation in PSC-UC patients.

We can hypothesize that the reduced uptake of bile acids in the terminal ileum occurring during cholestasis or the reduced bile acid secretion occurring in PSC could lead to a secondary feedback downregulation of colonic FXR; this would make more sense in advanced disease but unfortunately no data about PSC stage or serum bilirubin levels among the PSC-UC group was available.

Recent animal experiments have shown that activation of FXR in the intestine protects the liver from cholestasis by reducing the hepatic pool of bile acids.[27] Failure to activate FXR or constitutive downregulation of FXR could therefore exacerbate PSC and hepatic cholestasis by increasing bile acids levels within hepatocytes. In a recent study, 2355 IBD patients (1193 with UC) and 853 controls were genotyped with seven tagging SNPs and two functional SNPs for FXR. None of the SNPs was associated with the presence of IBD; however, no information or subanalysis for patients with PSC-UC was provided.[21] Ongoing[28] and future genome-wide association studies in PSC will probably shed some light on the role of FXR and other nuclear receptors and their role for PSC development and progression. It is apparent that the crosstalk between the liver and the colon in PSC-UC patients has yet to be explored and FXR may be just another piece of the puzzle.

ACKNOWLEDGMENT

The authors thank the pathology residents Mabel Ko and Jaclyn Hechtman for help with the tissue microarrays.

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THE FEATURES OF MUCOSA-ASSOCIATED MICROBIOTA IN PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

AP&T Alimentary Pharmacology and Therapeutics

The features of mucosa-associated microbiota in primary sclerosing cholangitis

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Publication data
Submitted 19 October 2015
First decision 12 November 2015
Resubmitted 23 December 2015
Resubmitted 14 January 2016
Accepted 18 January 2016
EV Pub Online 9 February 2016

This article was accepted for publication after full peer-review.

SUMMARY

Background
Little is known about the role of the microbiome in primary sclerosing cholangitis.

Aim
To explore the mucosa-associated microbiota in primary sclerosing cholangitis (PSC) patients across different locations in the gut, and to compare it with inflammatory bowel disease (IBD)-only patients and healthy controls.

Methods
Biopsies from the terminal ileum, right colon, and left colon were collected from patients and healthy controls undergoing colonoscopy. Microbiota profiling using bacterial 16S rRNA sequencing was performed on all biopsies.

Results
Forty-four patients were recruited: 20 with PSC (19 with PSC-IBD and one with PSC-only), 15 with IBD-only and nine healthy controls. The overall microbiome profile was similar throughout different locations in the gut. No differences in the global microbiome profile were found. However, we observed significant PSC-associated enrichment in *Barnesiellaceae* at the family level, and in *Blautia* and an unidentified *Barnesiellaceae* at the genus level. At the operational taxa unit level, most shifts in PSC were observed in *Clostridiales* and *Bacteroidales* orders, with approximately 86% of shifts occurring within the former order.

Conclusions
The overall microbiota profile was similar across multiple locations in the gut from the same individual regardless of disease status. In this study, the mucosa associated-microbiota of patients with primary sclerosing cholangitis was characterised by enrichment of *Blautia* and *Barnesiellaceae* and by major shifts in operational taxa units within *Clostridiales* order.

Aliment Pharmacol Ther 2016; **43:** 790–801

doi:10.1111/apt.13552

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic idiopathic cholestatic liver disease, characterised by progressive inflammation and fibrosis of bile ducts, strongly associated with inflammatory bowel disease (IBD), especially with ulcerative colitis (UC).[1–3] PSC-IBD patients have a distinctive phenotype as compared to IBD alone. They typically present asymptomatic or mildly symptomatic extensive colitis, a quiescent clinical course, more right-sided inflammation, rectal sparing, backwash ileitis, increased risk of pouchitis, and an increased risk for developing colorectal neoplasia.[4–10] Neither the cause of PSC,[11] nor the mechanisms by which IBD patients develop this unique phenotype when they have concomitant PSC are known.

Some data from basic and clinical studies support the hypothesis that the intestinal microbiota may have a role in disease pathogenesis.[12, 13] For example, animal models of surgically induced small intestinal bacterial overgrowth develop abnormalities in the intra- and extrahepatic bile ducts that resemble PSC by cholangiography and histology.[12] The improvement of liver enzymes following a trial of antibiotic therapy, and a recent case reporting laboratorial and histological abnormalities reversal following vancomycin treatment in a liver transplant PSC recurrence, also suggest a role for the gut flora in disease pathogenesis.[14–16]

With the advent of culture-independent techniques, a better understanding of how the gut microbiome can affect and modulate the development of liver diseases has emerged. Indeed microbiota alterations in cirrhosis are now well-documented.[17] A prior study has observed a lower abundance of uncultured *Clostridiales* in PSC patients as compared to IBD and healthy controls, not providing however more in-depth analysis.[18] Being a cholestatic liver disease with profound alterations in bile acid pool,[19] and acknowledging the reciprocal relationship between gut flora and bile acid metabolism,[20–23] it is very likely that patients with PSC-IBD exhibit alterations in the gut microbiota. The main objective of this study was to further explore the mucosa-associated microbiota in PSC patients, and to compare it with IBD-only and healthy controls.

MATERIAL AND METHODS

Ethical considerations

This study was approved by the Institutional Review Boards of Icahn School of Medicine at Mount Sinai and the University of Chicago. All patients and healthy controls signed an informed consent form.

Subjects and sampling

Between November 2011 and November 2014, patients with PSC, patients with IBD and healthy controls undergoing colonoscopy at the Mount Sinai Medical Center and at the University of Chicago, were prospectively recruited, within a collaborative multicentre programme for integrated studies in IBD [Sinai-Helmsley Alliance for Research Excellence (SHARE) Network]. Sample collection protocol was standardised across two institutions to avoid possible bias during sample collection and processing. The inclusion criteria were age greater than 18 years, confirmed diagnosis of PSC based on histology and/or abnormal cholangiogram (ERCP or MRCP), and confirmed diagnosis of IBD by conventional endoscopic and histological criteria.[24] Patients with a personal history of colectomy, a diagnosis of secondary sclerosing cholangitis, or concomitant infectious colitis at the time of colonoscopy were excluded. Patients with newly diagnosed PSC, who were scheduled for their initial colonoscopy to screen for IBD, and healthy controls undergoing screening colonoscopy, were also recruited.

Demographical and clinical information were recorded for every patient. During colonoscopy, disease severity was recorded, and biopsies for colorectal neoplasia screening were obtained, according to current guidelines. On all subjects, biopsies were collected from the left colon (LC) for microbiota analysis, and in a subset of patients, biopsies were also collected from the terminal ileum (TI) and right colon (RC) for comparison of the microbiota features across different colonic locations. Biopsies were either snap frozen or stored in RNAlater (Qiagen, Valencia, CA, USA) for subsequent analysis. All samples were analysed at Icahn School of Medicine at Mount Sinai, New York.

Tissue DNA extraction and 16S ribosomal RNA amplification

Tissue samples were transferred into bead tubes (MO-BIO, Carlsbad, CA, USA) and homogenised using bead beating method. Homogenised tissue samples were further processed using the Qiagen DNeasy Blood & Tissue Kit following the manufacturer's protocol (Qiagen). Total DNA concentration was determined with Qubit 2.0 Fluorometer (Life Technologies, Norwalk, CT, USA). The phylogenetically informative V3–V4 region of 16S ribosomal RNA (rRNA) gene was amplified using universal primer set 347F/803R.[25] The primers were synthesised by IDT (Integrated DNA Technology, Coralville, IA, USA). We used a dual-barcoding approach to label the 16S rRNA amplicons from each sample as described

previously. Briefly, the 6-mer barcodes were attached on the 5′ ends of both forward and reverse PCR primers so that 16S rRNA PCR amplicons from each sample contained a unique dual barcode combination. The 16S rRNA amplicons were further pooled with equal molarity and submitted for MiSeq 2 × 300 pair-end sequencing at high depth. The paired sequence readings were merged and filtered by size (>400 bp) and quality score (>Q30) using CLC genomics workbench version 7. The processed readings were further split by dual barcode for each sample and assigned taxonomic classification using QIIME pipeline 1.8.0.[26] Repeated measurements of the same sample were made to assess sequencing reproducibility. After processing, QIIME provided detailed operational taxa unit (OTU) tables containing the microbiota composition and abundance for each individual sample.

Metagenomic 16S rRNA data analysis. To characterise the gut microbiota, firstly the overall microbiota dissimilarities among all samples, also known as beta-diversity, were accessed using the Bray–Curtis distance matrices and visualised by nonmetric multiple dimensional scaling plots. The PerMANOVA test,[27] with the maximum number of permutations = 999, was performed using the [Adonis] function of the R package vegan 2.0-5[28, 29] to test the significance of the overall microbiota differences between groups by PSC and IBD status. Secondly, the diversity of the microbial community within each sample, so-called alpha-diversity, was measured using the Shannon Index as a metric to represent the species diversity.[30] Next, significant differential taxa features at the family and genus levels were selected using random forest algorithm, a supervised machine learning approach, using R *package* rfPermute and confirmed by Boruta feature selection (R *package* Boruta).[31, 32] Only features that were consistent in both analyses were selected. The significance of the selected taxa was further tested by *t*-test. In addition, at the OTU level, we performed the log likelihood ratio test (QIIME command group_significance.py using *G*-test statistics) to further identify significant differential OTUs between PSC and the healthy controls using LC samples only. The resulted *P*-values were adjusted by the FDR methods. We also compared the PSC vs. IBD-only at LC, RC and TI locations using the differential OTUs selected from LC samples.

Blautia-specific long 16S rRNA sequencing

Long 16S rRNA reads can further improve the taxa OTU inference.[33, 34] Therefore, we designed 16-base-barcoded 404F/1263R primer pairs (Table S1) specifically for the *Blautia* genus based on 16S rRNA reference sequence of the *Blautia* genus. The ~860 bp-sized PCR amplicons were pooled for sequencing on the PacBio RS II. Sequencing data from PacBio was processed using the manufacturer provided programme smrtanalysis v.2.1.1 (https://github.com/PacificBiosciences/SMRT-Analysis/). Circular consensus sequencing reads were then filtered by size (>800 bp) and the quality score (>Q30) using CLC genomics workbench version 7. After split by barcode for each sample, all filtered reads were processed using QIIME pipeline 1.8.0.[35] The generated OTUs were filtered to only keep OTUs assigned to the *Blautia* genus and with more than 100 counts of reads. We performed the *G*-test of independence[35] to determine whether *Blautia* OTU presence/absence is associated with PSC status at LC samples only, in which we combined healthy control and non-PSC IBD together as non-PSC group to compare with PSC. Representative sequences from significantly differential *Blautia* OTUs were further aligned with the *Blautia* reference sequences to construct the maximum likelihood phylogenetic tree using UPGMA method and perform a pair-wise sequence alignment comparison.

RESULTS

Study population

Between November 2011 and November 2014, 46 patients were enrolled at both centres: 20 with PSC (19 of which had concomitant IBD), 16 with IBD and 10 healthy controls. There were 27 males (61%) and the median age for the whole population was 47 years (IQR 33.5–58). The mean age of each group was as following: 43.8 years for healthy controls, 50.3 for the IBD group and 45.3 for the PSC group. No patient was on antibiotics at the time of colonoscopy.

Samples from two subjects (one IBD patient and one healthy control) were eliminated from further analysis due to over-contamination (>90% relative abundance with *Escherichia coli*). Therefore, 44 patients were included in the final analysis: 20 patients with PSC (19 with PSC-IBD and one with PSC-only), 15 patients with IBD and nine healthy controls (Table 1). Biopsies from different colonic locations (TI, RC and LC) were available in 18 subjects (11 with PSC, six with IBD-only and one with PSC only), except for two cases, where the TI could not be intubated. The clinical characteristics of PSC and IBD patients are described in Table 1. The patient with PSC-only was analysed in the PSC group.

Table 1 | Clinical characteristics of PSC and IBD (the patient presenting PSC-only was included in the PSC group together with PSC-IBD)

	PSC (n = 20)	IBD (n = 15)
Male (n, %)	16 (80)	9 (60)
Age (years)		
Median (IQR)	47 (33.5–59.3)	48 (34.5–59.5)
Smoking status (%)		
Never	13 (65)	10 (67)
Ever	6 (30)	5 (33)
Unknown	1 (5)	
Type of IBD		
UC/IBDU	13 (65)	13 (87)
CD	6 (30)	2 (13)
No IBD	1 (5)	
Extent of IBD	UC/IBDU Extensive colitis – 100% CD Colonic disease: 3 (50%) Ileocolonic disease:3(50%)	UC Extensive colitis – 12 (92%) Left colitis – 1 (8%) CD Colonic disease: 2 (100%)
PSC duration; median years (IQR)	4 (2–12.3)	–
IBD duration, median years (IQR)	9 (4.8–18.9)	9 (4.75–22)
PSC Mayo score*, median (IQR)	0.03 (−0.63 to 0.42)	–
Endoscopic score of inflammation†		
Normal/quiescent	11	11
Mild	5	3
Moderate	4	1
Medications at the time of colonoscopy		
Unknown/No medications	5	–
5-ASA	8	10
Thiopurines	5	8
Anti-TNF	5	3
Ursodeoxycholic acid	10	–
Tacrolimus	1	–
Cholestyramine	1	–

IQR, interquartile range; IBDU, IBD unclassified.

* The PSC Mayo score could not be calculated in two patients due to lack of information.

† For purposes of simplicity the endoscopic score CD-SES was replaced in this table by the subjective impression of the endoscopist performing the colonoscopy into normal, mild or moderate inflammation (the median CD-SES for the patients with CD in this study was 11.5, IQR 10–13).

One of the PSC-IBD patients had a history of liver transplant and another had a history of choledochojejunostomy for a dominant stricture. There was no history of colon surgery among any of the participants.

Samples

16S rRNA amplicons from 81 samples (44 LC, 18 RC, 16 TI and three technical repeats) were sequenced and a total of 9.3 million reads were generated after filtering by size and quality, as described in the methods section. On average, each sample contained ~110 000 reads. The repeated measurements showed Pearson correlation with be 99% at the genus level. We used the mean of the repeated measurements for further analysis.

The mucosa-associated microbiota is stable across different locations within each individual

Taking into account, the distinct phenotype of more inflammation and a higher prevalence of neoplasia in the RC observed in PSC-IBD patients, we specifically assessed if there were any differences in the spatial distribution of the mucosa-associated microbiota between the RC, LC and TI. Therefore, we compared the microbiota composition at three biopsy locations in the 18

subjects, from whom multiple locations were available. Our results (Figure 1a) showed that although a few samples from the same subjects showed substantial variations, in general, the overall microbiota was consistent across all three sampling locations. The distance across locations within the same subject was significantly smaller than the distance between samples from the same location but from different subjects (mean = 0.18 and 0.45, s.d. = 0.13 and 0.17, respectively, $P < 0.05$). No significant differences in the species richness (measured by the Shannon index) were observed between PSC and IBD-only patients in all three locations (Figure 1b).

PSC associated LC mucosa microbiota features

Observing that there were no overall differences in the microbiota diversity between TI, RC and LC, we next analysed the microbiota composition only in the LC of all subjects. The overall microbiota dissimilarities among all 44 LC samples (20 PSC, 15 IBD patients and nine

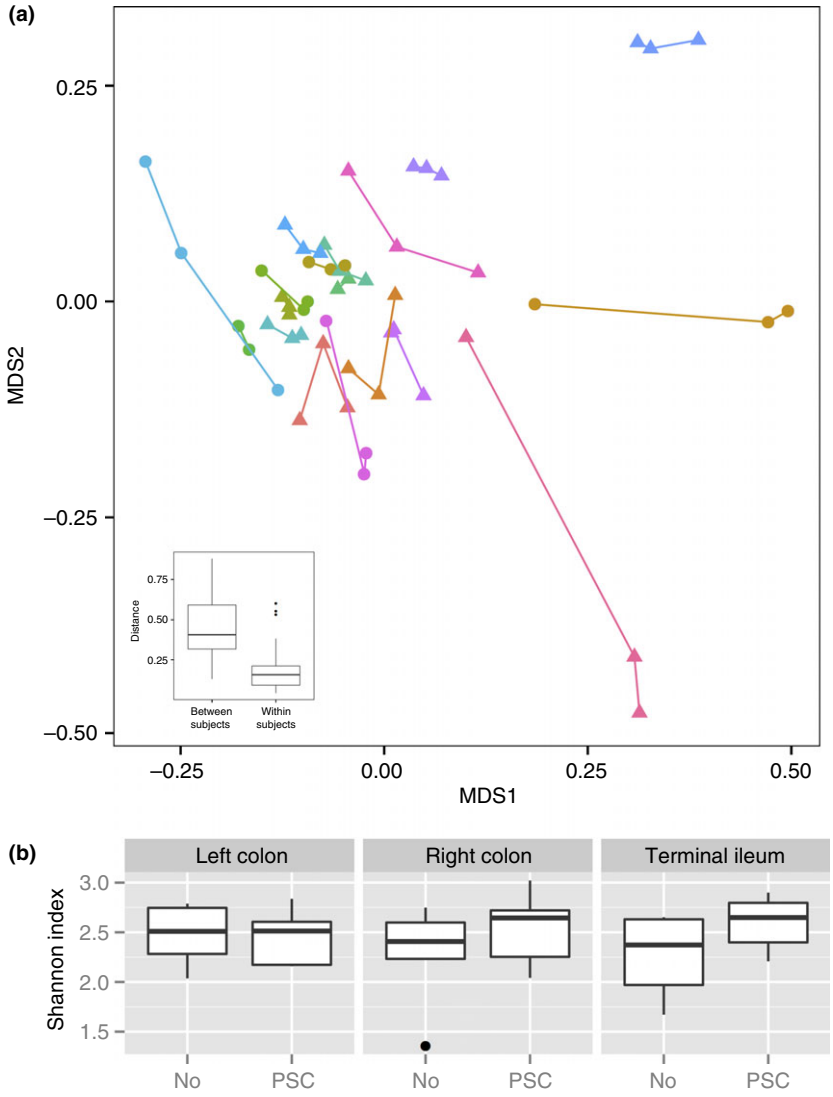

Figure 1 | Microbiota profiles across multiple biopsy locations. (a) Overall microbiota dissimilarities between samples. Dissimilarities were measured by distance and visualised using nonparametric multidimensional scaling plot. Lines link the multiple sampling locations from the same subject. Insert at lower left corner shows the mean and variance of the distance between the samples from the same or different subjects. Triangles represent PSC patients; Circles represent non-PSC, IBD patients. (b) The boxplots show the mean and variance of the richness of the microbial community within each sample, showing no significant difference across multiple locations.

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healthy controls) grouped by PSC and IBD status were accessed using the Bray–Curtis distance matrices (Figure 2a). Although we noticed a separation between the healthy control and the IBD or PSC-IBD, the PERMANOVA test did not find a significant difference in the global LC microbiota profile by disease status. We also did not observe a significant difference among controls, IBD and PSC samples in species richness using Shannon Index (Figure 2b). At the taxa level, two families including *Barnesiellaceae* and *Alcaligenaceae,* as well as two genera including *Blautia* and an unidentified genus from *Barnesiellaceae* family were selected by random forest algorithm using R package rfPermute and confirmed by Boruta feature selection (R package Boruta) (Figure 2c). Among those selections, we observed significant enrichment of *Barnesiellaceae* family and its further assigned unidentified genus (mean abundance = 1.3% in PSC samples, 0.48% in IBD and 0.16% in healthy controls; $P = 0.44$, 0.025, respectively by *t*-test) and *Blautia* (mean = 4.5% in PSC samples, 2.9% in IBD and 2.1% in healthy controls; $P = 0.22$ and 0.02, respectively by *t*-test) in PSC samples compared to healthy controls. To test whether or not the PSC-associated taxa features found in LC were consistent in the RC and TI, we further compared the abundance of *Barnesiellaceae* family and *Blautia* genus at three locations in the available samples from these locations. We found that not only did the enrichment of both taxa in PSC

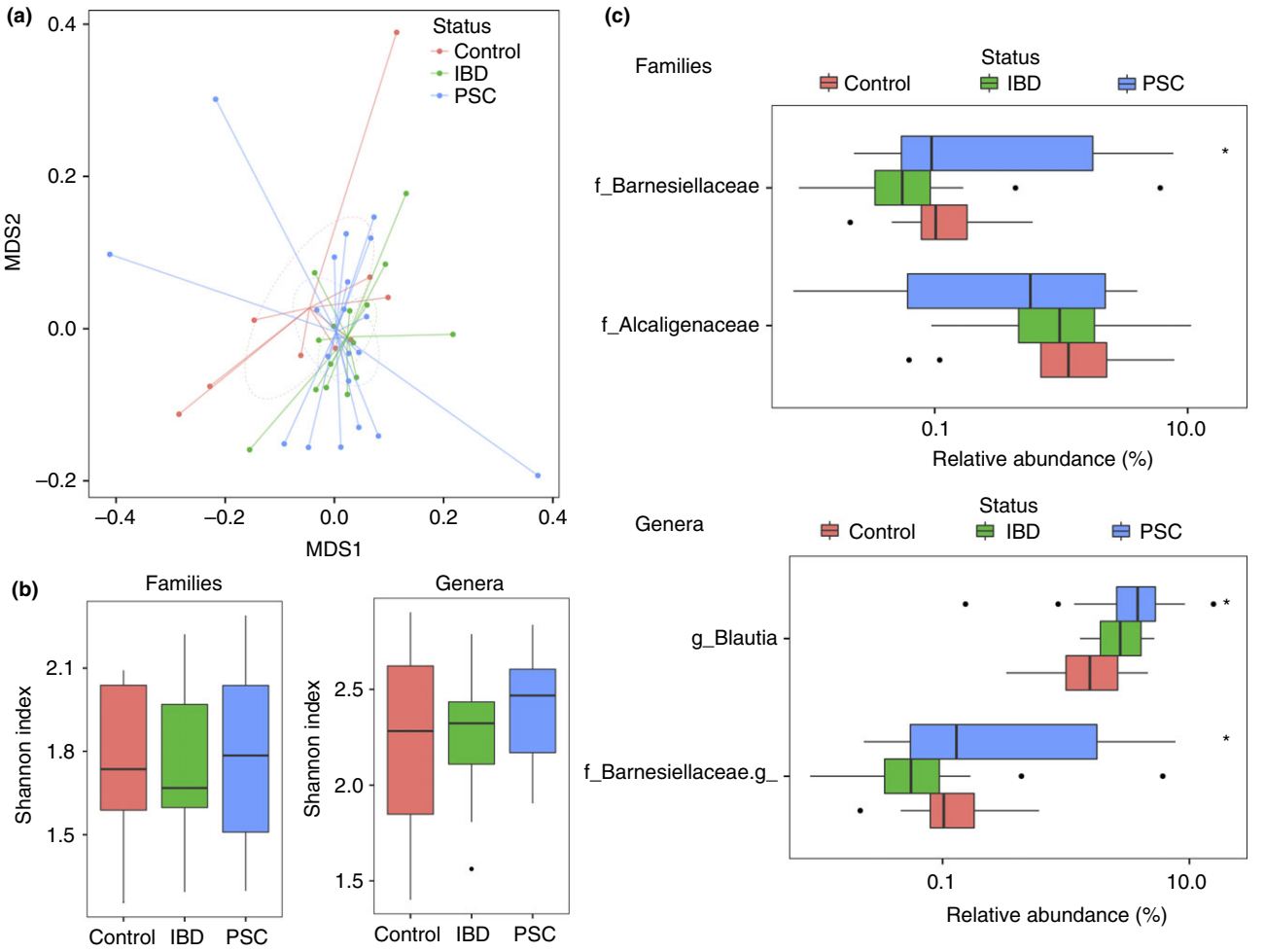

Figure 2 | Microbiota dissimilarities by PSC and IBD status in left colon samples. (a) Overall microbiota dissimilarities between samples grouped by PSC and IBD status. Dissimilarities were measured by distance and visualised using nonparametric multidimensional scaling plot. (b) The boxplots show the mean and variance of the richness of the microbial community between different disease status. (c) The log-scaled boxplots show the differential taxa features selected at family and genus level by health status. The asterisk indicates the *P* values < 0.05 between PSC and healthy controls.

patients occur in all locations but also that the abundance of those taxa was consistent across multiple locations within the same individual (Figure 3). Further analysis excluding the patients with PSC with an history of OLT, and an history of choledochojejunostomy did not change results (data not shown).

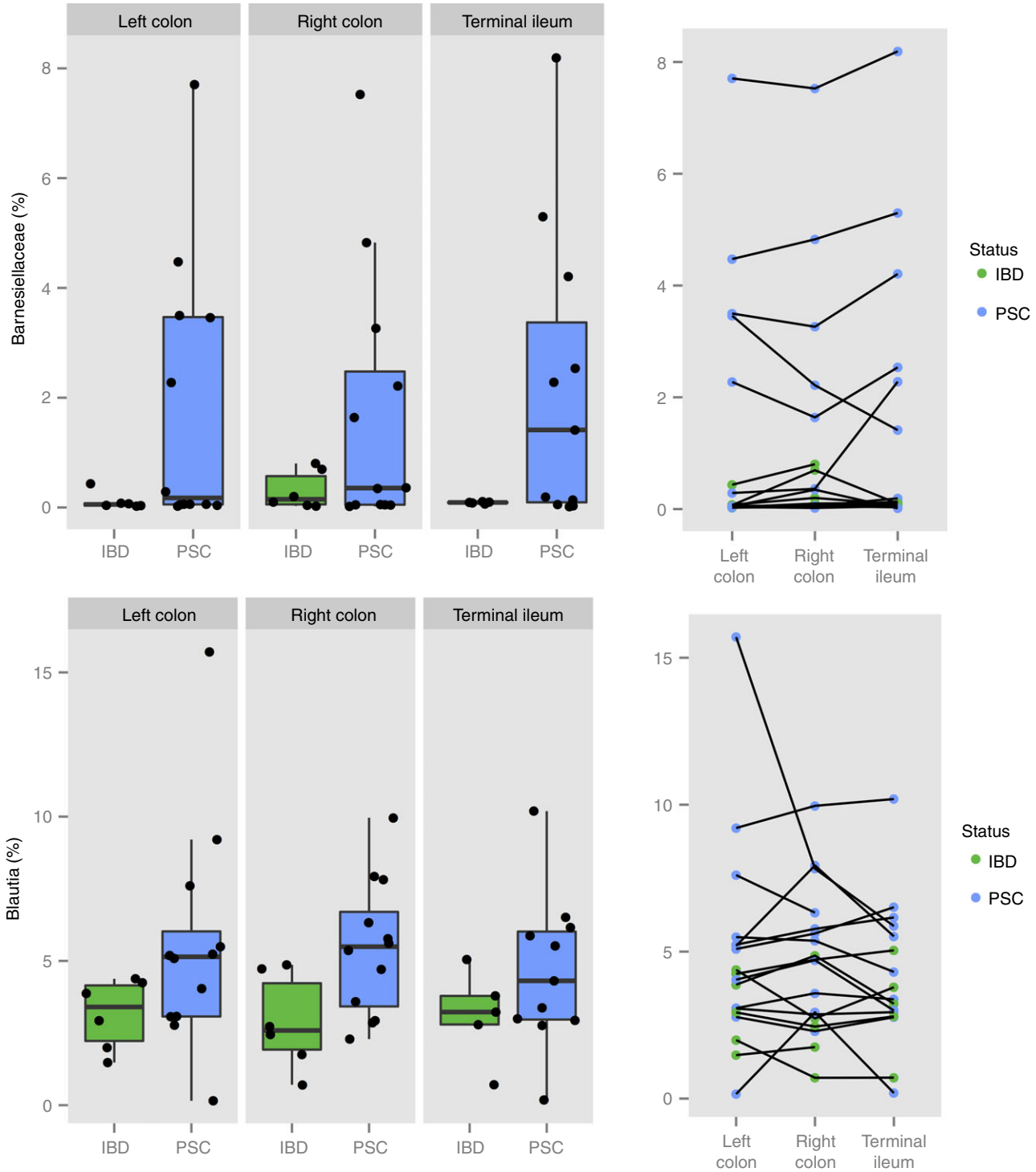

Figure 3 | The relative abundance of *Barnesiellaceae* and *Blautia* in PSC and non-PSC-IBD at multiple locations. Boxplots (left panel) show the mean and variance of the relative abundance; Dot plots (right panel) show the relative abundance for each individual samples and the lines link the samples from the same subject. Green: non-PSC IBD; blue: PSC.

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We then assessed whether there were any differences in microbiota composition according to PSC severity score. Based on the ranked PSC Mayo score, the PSC patients were assigned to low risk (Mayo score <0), intermediate risk (Mayo score from 0 to 2) and high-risk score (Mayo score >2). There were no patients with severe disease; however, among the low and intermediate risk, groups had a similar global microbiota composition (Figure S1a) and taxa richness (Figure S1b). At the taxa level, the level of *Blautia* was not different between two risk groups. The low risk group showed higher median level of *Barnesiellaceae* family compared to the intermediate risk group (Figure S1c), but this did not reach statistical significance.

Differential OTUs by PSC status

We performed *de novo* OTU picking using QIIME pipeline. Based on the 97% similarity of the 16S rRNA sequencing reads, all sequencing reads were clustered into individual OTUs. After removing rare OTUs (relative abundance <0.1% in all samples), we compared 2439 OTUs and selected 80 and 15 OTUs significantly ($P < 0.05$ by parametric *t*-test, not adjusted) different between PSC, healthy control (Table S2) or IBD (Figure S2). We found that when compared with healthy controls, most of the PSC-associated shifts in the bacterial composition were observed in the *Clostridiales* and *Bacteroidetes* orders, with 86% in the former order. In agreement with our above findings, several PSC-enriched OTUs belonged to the *Blautia* genus and the *Barnesiellaceae* family. When compared to IBD, many OTUs were from the *Blautia* genus. Similar enrichments and reductions at the PSC-associated OTUs selected from LC samples (Figure S2) were observed in RC and TI locations.

Differential OTUs at Blautia genus between PSC and non-PSC by long-read 16S rRNA sequencing

Our results showed that both *Barnesiellaceae* and *Blautia* genus were enriched in PSC patients. But unlike *Barnesiellaceae*, *Blautia* comprised >2% of the entire microbiota regardless of the disease status, so it is plausible to specifically enrich this genus and further use the long 16S rRNA reads to perform additional taxa OTU inference at *Blautia* genus in both patient and control samples. After processing, 2967 OTUs were assigned to *Blautia* genus. Among those, 135 OTUs had reads of more than 100 counts. The *G*-test of independence further selected seven of 135 OTUs significantly different between PSC and non-PSC (unadjusted $P < 0.05$). We aligned those *Blautia* OTUs with *Blautia* reference sequences and constructed the maximum likelihood phylogenetic tree using UPGMA method (Figure S3a). We also performed the pair-wise sequence alignment comparison and showed the number of difference (upper) and the per cent identity (lower) between pairs (Figure S3c). Our results showed that four OTUs (denovo17640, denovo28452, denovo13317, denovo6236) were enriched and three OTUs (denovo25871, denovo781, denovo18792) were reduced in PSC samples (Figure S3b). We also found that the OTUs denovo17640, denovo13317 and denovo28452 showed >99% identity to reference strains from species of *Blautia wexlerae*, *Ruminococcus obeum* and *Blautia faecis*, respectively (Figure S3c).

DISCUSSION

Herein, we compared the mucosa-associated bacterial flora of patients with PSC to IBD alone and to normal subjects. As faecal microbiota does not necessarily reflect the mucosa-associated microbiota,[36, 37] we chose to examine the mucosa-associated microbiota, rather than stool, to gain more insight into the unique phenotype of PSC-IBD patients who tend to develop more right-sided inflammation, and proximal neoplasia. We thought there might be some differences in the microbiota composition throughout different locations of the ileo-colon in this specific clinical context, given the phenotypic associations with disease location for PSC.[5] However, and in line with previous observations in healthy subjects,[38] no significant site-specific differences in the microbial composition were found throughout the colon. Although this analysis may have been limited by sample size, it is possible that other mechanisms may be operating and interacting differently with the microbiota in the right as opposed to the LC[39] in patients with PSC and IBD. Rossen *et al.*[18] in a prior study, also described similar hierarchical clustering between samples from the TI and RC from the same subject from between PSC, IBD and healthy controls. However, no comparisons with the mucosa-associated from the LC were available. The same authors described a lower diversity and abundance of uncultured *Clostridiales II* at the genus level compared to UC and healthy controls. However, this study, was limited by a smaller sample size and, by the use of a probe-based approach, the HITChip[18, 40] that only allows detection of phylotypes present in the chip at the genus-like level. Our study, using a longer read 16S sequencing, allowed us to provide deeper taxonomic

analysis that could inform on specific microbiome shifts associated with PSC. Another recently published study,[41] assessing differences in microbial composition PSC-UC and UC patients, did not report differences in overall microbial diversity between PSC-IBD and UC, however different geographical provenience of participating individuals, was the main driver for microbial composition in this study. We observed a trend in the compositional dissimilarity of the overall microbiota between healthy controls, and PSC-IBD, but this did not reach statistical significance. This could be due to the modest sample size, underrepresentation of healthy controls, and disease remission for most of the IBD patients, since shifts in the microbiota can vary according to disease activity.[42] Using bacterial 16S rRNA next-generation sequencing, we found, across all colonic locations, a consistent PSC-enrichment in *Blautia and Barnesiellaceae* genera and shifts under the *Clostridiales*, and less frequently under the *Bacteroidales* order. Deeper taxa analysis at the OTU level was consistent with these findings, also showing several enriched OTUs particularly from *Blautia* and *Barnesiellaceae* genera. Specifically, around 86% of the relative changes in the microbiota occurred within the *Clostridiales* order, with reduction in three and enrichment in 66 OTUs. This is interesting, as shifts in these taxa have been observed both in IBD and also in cirrhosis.[20, 43] *Bacteroidetes* species play an important role in protein metabolism[44] as well as in bile acid deconjugation.[45] The *Clostridiales* order encompasses bacteria from *Lachnospiraceae* family, *Ruminococcaceae* family *and Blautia* genus, which are able to perform 7α-dehydroxylation,[23] an important step in converting primary to secondary bile acids in the intestine. Furthermore, it has been demonstrated that certain *Clostridum* spp. can affect number, function and differentiation of colonic Treg cells, therefore playing a crucial role in colonic homeostasis.[46] In advanced cirrhosis, a shift in the gut flora towards the enrichment of *Enterobacteriaceae* and the reduction in *Clostridiales* and *Bacteroidetes* in parallel with a reduced level of faecal secondary bile acids has been described.[20] Conversely, Islam and colleagues showed that feeding animal models with bile acids leads to an enrichment of colonic bile acids, which in turn results in the expansion of the *Firmicutes* phylum, specifically within the *Clostridia* class, with *Blautia spp* expanding significantly.[47] Being a cholestatic liver disease, characterised by scarring of the bile ducts, PSC is expected to lead to a reduction in the flow of bile acids from the liver to the intestine, at least in the more advanced stages of the disease. However, there also are data suggesting that during obstructive cholestasis, the apical sodium dependent bile acid transporter ASBT is down-regulated,[48, 49] as a feedback anti-cholestatic mechanism. This could hypothetically lead to a relative increase in BA in the proximal colon, which could in turn lead to an enrichment of bacterial species involved in bile acid handling.[23, 48] Since in our study, we did not have patient′s bile acid profiles we could not test for this hypothesis.

A limitation of our study is its relatively small sample size, so we were unable and unpowered to make any associations between disease severity and microbial composition, or to take into account any impact of medication, diet or disease course. Despite this limitation, and compared with previous studies, we were able to compare microbiota composition from multiple sites,[41] had representation of PSC-IBD patients, IBD and normal controls,[41] and a reasonable sample size,[18] that allowed to perform deeper taxonomic analyses.[18] We observed that the mucosa-associated microbiota was consistent among all locations, and that the *Blautia* and *Barnesiellaceae* enrichment was consistently found not only in the LC samples but also in other locations. Consistent with findings at the family and genus level, deeper OTU level analyses also found enrichment of *Blautia* and *Barnesiellaceae* OTUs in PSC. Therefore, in that sense we validated our findings. The cross-sectional nature of the study does not allow us to conclusively determine a causal link between the abundance of these species and its role in PSC. It is possible that the shifts in the microbiota features we observed in PSC-IBD are a consequence, rather than the cause, of the interaction between cholestasis and colonic inflammation. Furthermore, in the absence of a non-PSC liver disease control group, it is difficult to appreciate if the microbiota shifts we observed are specific to PSC or belong to a broader dysbiosis observed in chronic liver diseases.[17, 20] Whether these changes contribute to the special phenotype observed in PSC-IBD patients can only be speculated upon at this stage, and merits further investigation. Future studies investigating the role of microbiota in PSC should aim at collecting larger samples sizes, that could allow adjustment for clinical and analytical variables that could influence microbial composition in PSC such disease duration and stage, medication intake, diet, geographical location of patients, impact of liver transplant and or biliary surgery, as well as serum and faecal bile acid pool.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Microbiome profiles and PSC disease severity. (a) Overall microbiome dissimilarities between samples with low-risk severity and intermediate risk severity as assessed by the PSC Mayo score. (b) The boxplots show the mean and variance of the richness of the microbial community within each sample, showing no significant difference according to disease severity. (c) The boxplots show the mean and variance of the relative abundance of *Blautia* and *Barnesiellaceae* in patients with low-risk and intermediate risk PSC.

Figure S2. The fold changes and relative abundance of selected differential OTUs in PSC vs. non-PSC IBDs in the LC, RC and TI biopsies. Bar plots show the fold changes of the relative abundance of each select OTUs in PSC vs. non-PSC IBDs. Red color indicates the enrichment in PSC. Green color indicates the reduction in PSC. The mean abundance in PSC, non-PSC IBD and healthy controls is shown on the right. red: healthy control; green: non-PSC IBD; blue: PSC.

Figure S3. The maximum likelihood phylogenetic tree within *Blautia* genus using UPGMA method. We combined seven significantly PSC-associated OTUs and 15 reference sequences to construct the tree. The colored bar plots present the relative abundance of selected OTUs in PSC and non-PSC (combined healthy control and non-PSC IBDs) subjects. The table showed the number of differences (upper) and the per cent identity (lower) calculated from pair-wise sequence alignment comparison. The color gradient from blue to red indicates the low to high sequence similarity.

Table S1. 16S sequencing PCR primer sequences. The sequences of dual-barcoding 16S PCR primers targeted on 460 bp amplicons for Miseq sequencing. The primer sequences include 2 bp linker (italicised) and 6 bp barcodes (bold) at the 5′ end.

Table S2. The fold changes and relative abundance of selected differential OTUs in PSC vs. healthy controls in the left colon biopsies. Table columns represent mean of the selected OTUs in each study group: controls, IBD and PSC. The ratio between mean_OTUs in OSC and controls is represented with its corresponding taxonomy and *P* value.

AUTHORSHIP

Guarantor of the article: Jianzhong Hu.

Author contributions: JT, JFC, JH and SI have contributed to the study design and concept. All authors have contributed to the acquisition of data and have reviewed versions of the manuscript and provided critical comments. JT, JH and SI analysed, interpreted the data and drafted the manuscript. SI, BJ, JH obtained funding support.

ACKNOWLEDGEMENTS

We thank the OCS genome technology centre of New York University Langone Medical Center for the Illumina Miseq library preparation and sequencing service. We thank the genomic core facility at Icahn School of Medicine at Mount Sinai for the long-read 16S rRNA sequencing using the Pacific Biosciences RS II for single molecule sequencing.

Declaration of personal interests: Joana Torres has received speaking fees from Falk and consulting fees from Abbvie. Joel Pekow had received funding from NIH, the American Cancer Society, Takeda, and Abvie. Joseph Odin is an advisory board member for Intercept Pharmaceuticals and has received research funding from Gilead, NIDDK. J.-F. Colombel has served as consultant, advisory board member or speaker for Abbvie, AB Science, Amgen, Bristol Meyers Squibb, Celltrion, Danone, Ferring, Genentech, Giuliani SPA, Given Imaging, Janssen, Immune Pharmaceuticals, Medimmune, Merck & Co., Millenium Pharmaceuticals Inc., Neovacs, Nutrition Science Partners Ltd., Pfizer Inc. Prometheus Laboratories, Protagonist, Receptos, Sanofi, Schering Plough Corporation, Second Genome, Shire, Takeda, Teva Pharmaceuticals, Tigenix, UCB Pharma, Vertex, and Dr. August Wolff GmbH & Co. Steven Itzkowitz received grant support and fees for serving on a scientific advisory board from Exact Sciences Corporation.

Declaration of funding interests: This study was supported by funds from National Institute of Health, NIDDK 1K01DK094986-01(JH); The Chemotherapy Foundation (SI) and The Helmsley Foundation (SI, BJ, IP).

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THE GUT MICROBIOTA, BILE ACIDS AND THEIR CORRELATION IN PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

UNITED EUROPEAN GASTROENTEROLOGY ueg journal

Original Article

United European Gastroenterology Journal
2018, Vol. 6(1) 112–122

DOI: 10.1177/2050640617708953
journals.sagepub.com/home/ueg
SAGE

The gut microbiota, bile acids and their correlation in primary sclerosing cholangitis associated with inflammatory bowel disease

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Abstract

Background: Patients with primary sclerosing cholangitis associated with inflammatory bowel disease (PSC-IBD) have a very high risk of developing colorectal neoplasia. Alterations in the gut microbiota and/or gut bile acids could account for the increase in this risk. However, no studies have yet investigated the net result of cholestasis and a potentially altered bile acid pool interacting with a dysbiotic gut flora in the inflamed colon of PSC-IBD.

Aim: The aim of this study was to compare the gut microbiota and stool bile acid profiles, as well as and their correlation in patients with PSC-IBD and inflammatory bowel disease alone.

Methods: Thirty patients with extensive colitis (15 with concomitant primary sclerosing cholangitis) were prospectively recruited and fresh stool samples were collected. The microbiota composition in stool was profiled using bacterial 16S rRNA sequencing. Stool bile acids were assessed by high-performance liquid chromatography tandem mass spectrometry.

Results: The total stool bile acid pool was significantly reduced in PSC-IBD. Although no major differences were observed in the individual bile acid species in stool, their overall combination allowed a good separation between PSC-IBD and inflammatory bowel disease. Compared with inflammatory bowel disease alone, PSC-IBD patients demonstrated a different gut microbiota composition with enrichment in *Ruminococcus* and *Fusobacterium* genus compared with inflammatory bowel disease. At the operational taxonomic unit level major shifts were observed within the *Firmicutes* (73%) and *Bacteroidetes* phyla (17%). Specific microbiota-bile acid correlations were observed in PSC-IBD, where 12% of the operational taxonomic units strongly correlated with stool bile acids, compared with only 0.4% in non-PSC-IBD.

Conclusions: Patients with PSC-IBD had distinct microbiota and microbiota-stool bile acid correlations as compared with inflammatory bowel disease. Whether these changes are associated with, or may predispose to, an increased risk of colorectal neoplasia needs to be further clarified.

Keywords

Gut microbiota, bile acids, primary sclerosing cholangitis, inflammatory bowel disease

Received: 14 October 2016; accepted: 12 April 2017

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Key summary

Summary of the established knowledge on this subject

1. Primary sclerosing cholangitis is a chronic cholestatic disease of unknown etiology and frequently associated with inflammatory bowel disease.
2. Emerging evidence suggests that alterations in the microbiome may be associated with this special phenotype.
3. No studies have yet investigated the net result of cholestasis and a potentially altered BA pool interacting with a dysbiotic gut flora in the inflamed colon of PSC-IBD.

What are the significant and/or new findings of this study?

1. Patients with PSC-IBD presented demonstrated a different gut microbiota composition, and specific microbiota-fecal BA correlations.
2. Despite no significant differences in the specific BA in stool, the overall combination of stool BA was discriminant between PSC-IBD and IBD.

Introduction

Primary sclerosing cholangitis (PSC) is a rare chronic cholestatic liver disorder of unclear aetiology.[1] It is characterized by chronic inflammation of the biliary epithelium, that eventually leads to fibrosis, resulting in multifocal strictures of the intrahepatic and extrahepatic bile ducts. It can lead to cirrhosis, and end-stage liver disease requiring orthotopic liver transplantation (OLT).[1] Furthermore, PSC is also associated with an increased risk of cholangiocarcinoma, gallbladder cancer and colorectal cancer.[2,3] The strongest risk factor for having PSC is a history of inflammatory bowel disease (IBD). Around 60–80% of PSC patients will also have IBD, most commonly ulcerative colitis (UC). Ironically, despite having mild or quiescent extensive colitis, patients with PSC-IBD have the highest risk of developing colitis-associated neoplasia, which, in comparison with IBD, tends to be located preferentially in the right side of the colon.[2,4] The factors that contribute to the increased risk of colorectal neoplasia in PSC remain unknown.[5,6] A potential role for altered luminal concentration and/or composition of secondary bile acids (BAs) has been suggested, but never confirmed.[7] Data from basic and clinical studies have long supported the hypothesis that the intestinal microbiota may have a role in PSC pathogenesis.[8–10] Recently, studies using next-generation sequencing have reported a distinct fecal or mucosal microbiota composition in PSC-IBD patients.[11–18] There is a close interplay between gut flora and BA metabolism. Besides their role in nutrient absorption and lipid digestion, BAs are important signaling molecules, acting in inflammation and metabolism, through activation of BA receptors such as the G-protein-coupled BA transmembrane receptor TGR5, and the nuclear BA receptor Farnesoid X receptor (FXR).[19] BAs have antimicrobial properties, and through FXR-activation they regulate the expression of host genes whose products promote innate defence against luminal bacteria.[20,21] On the other hand, BA metabolism is a property of the gastrointestinal microflora; BAs are transformed from primary BA (cholic acid (CA) and chenodeoxycholic acid (CDCA)) to secondary BA (litocholic acid (LCA) and deoxycholic acid (DCA)) by deconjugation, 7-alpha de-hydroxylation and epimerization (CDCA → ursodeoxycholic acid (UDCA)) by the gut microbiota; therefore the degree of activation of the BA receptors is also largely influenced by the gut microbiota.[22–24] Nothing is known about the net result of cholestasis associated with PSC, and a potentially altered BA pool interacting with a dysbiotic gut flora in the inflamed colon of PSC-IBD patients. In this article, we have explored the BA profiles, the gut microbiota, and their correlation in PSC-IBD as compared with IBD patients.

Methods

Ethical considerations

This study was approved by the Portuguese National Committee for Data Protection and the local ethics committee. All patients signed an informed consent form.

Subjects and samples

Between October 2014–July 2015, 15 patients with PSC-IBD and 15 patients with IBD were prospectively recruited. The inclusion criteria were age greater than 18 years old, confirmed diagnosis of PSC based on histology and/or abnormal cholangiogram (Endoscopic Retrograde Cholangio-Pancreatography or Magnetic resonance cholangiopancreatography), a confirmed diagnosis of IBD by conventional endoscopic and histological criteria, and the presence of extensive colitis. Patients with a personal history of colectomy, a

diagnosis of secondary sclerosing cholangitis or a history of OLT were excluded. All patients provided clinical and demographic information, and completed a semi-quantitative food frequency questionnaire (FFQ) validated for the Portuguese population.[25] Clinical activity was scored according to the Mayo score for ulcerative colitis,[26] and the Harvey-Bradshaw index for Crohn's disease.[27] Endoscopic activity was scored according to the Mayo endoscopic score for UC[26] and the Simple Endoscopic Score for Crohn's Disease (SES-CD) for CD.[28] All study participants collected serum sample, and a stool sample for BA analysis and microbiota analysis. All PSC patients on UDCA therapy were required to stop it two weeks before specimen collection. A minimum interval of three months was required between antibiotic intake or bowel preparation (for colonoscopy) and sample collection. During colonoscopy disease severity was recorded, and biopsies for colorectal neoplasia screening were obtained, according to current guidelines.

Serum BA profiles

A fasting serum sample was obtained from each patient. Individual amidated BAs in serum (1 ml) were determined by high-performance liquid chromatography (HPLC),[29] after solid-phase extraction using Sep-Pak C18 cartridges (Waters Corp., Milford, Massachusetts, USA).[30] Only the conjugate fraction of BAs was measured in serum.

Stool BA profiles

A morning stool sample was obtained and dried to obtain a lyophilized extract. To lyophilized faecal samples weighing 1 g, 80% methanol was added. All samples were sonicated for 30 min, refluxed for two hours, and then cooled and filtered.[31] The residue was re-suspended in chloroform/methanol (1:1, v/v), refluxed for one hour, and filtered. The combined extracts were taken to dryness, and re-suspended in 10 ml methanol (MeOH). An aliquot of 1 ml was added with 2 µl of 1 mg/ml nordeoxycholic acid, and was diluted in 10 ml deionized water and deposed on a 300 mg HLB Oasis column, washed with 10 volumes deionized, 1 volume cyclohexane, and the BAs were then eluted with 5 ml MeOH and were taken to dryness and resuspended in 250 µl MeOH. Four microliters were injected on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) as previously described.[32] Results are reported in nmol/g of dried stool for total BAs and in proportion of the median after calibration of the method, with weighted mixtures and normalization relative to the internal standard.[32] The conjugate and non-conjugated species were quantified.

BA analysis

BAs were not normally distributed according to the Shapiro-Wilk test; therefore, their distributions were compared using non-parametric tests. The relative proportion of a given BA corresponds to its concentrations divided by the total of BAs. BA results are presented as the median proportion. For example, the total primary stool BA is the sum of CA and CDCA and their respective glyco-, tauro-, and sulphoderivatives. Linear discriminant analysis (LDA) was conducted to illustrate the classification of disease groups (IBD only and PSC-IBD) using stool BAs. LDA is a dimension reduction statistical technique that looks for a combination of features (continuous variables) that maximize the separation between classes. LDA was performed using the MASS package in R software.

Stool DNA extraction

Approximately 200 mg of stool were transferred into bead tubes (MO-BIO, Carlsbad, California, USA) and homogenized using the bead-beating method. Homogenized stool samples were further processed using the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol (Qiagen, Valencia, California, USA). Total DNA concentration was determined with Qubit 2.0 Fluorometer (Life Technologies, Norwalk, Connecticut, USA).

16S ribosomal RNA (rRNA) sequencing

The phylogenetically informative V3–V4 region of 16S ribosomal RNA (rRNA) gene was amplified using universal primer set 347F/803R.[33] The primers were synthesized by IDT (Integrated DNA Technology, Coralville, Iowa, USA). We used a dual-barcoding approach to label the 16S rRNA amplicons from each sample as described previously.[34] The 16S rRNA amplicons were further pooled with equal molarity and submitted for MiSeq 2×300 pair-end sequencing at high depth. The paired sequence readings were merged and filtered by size (>400 bp) and quality score (>Q30) using paired-end assembler for DNA sequences (PANDAseq).[35] The processed readings were further split by dual barcodes for each sample and assigned taxonomic classification using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline 1.9.0.[36] Repeated measurements of the same sample were made to assess sequencing reproducibility. After processing, QIIME provided detailed OTU tables containing the microbiota composition and abundance for each individual sample.

Data analysis

First, we measured the diversity of the overall microbiota communities within or across each sample. The overall species richness within each patient group, so-called alpha-diversity, was measured using the Chao1 and Shannon Index on rarefied tables at 8000 sequences per sample.[37] Beta-diversity was measured using unweighted and weighted UniFrac distance matrices on the rarefied tables. The permutational analysis of variance (PERMANOVA) test (number of permutations = 999), was performed using QIIME command compare_categories.py to test the overall microbiota differences between groups by PSC and IBD status.[38] Secondly, at the taxa level, the LDA effect size (LefSe) analysis was used with default parameters to select taxa features from phylum to genus level that were associated to PSC status.[39] Only features with LDA score >2.0 were kept. A Kruskal-Wallis test on the LefSe selected differential taxa at the genus level was performed, and corresponding *p*-values were adjusted for multiple comparisons. Finally, the Kruskal-Wallis test was also performed at individual OTUs to select OTUs with significant differential abundance with respect to the PSC-IBD status. All singleton OTUs were removed prior to all analysis.

Correlation networks

We calculated both Pearson's and Spearman's correlations between the most abundant (mean relative abundance >0.1%) 65 genera in the gut microbiota and the stool BA levels in PSC and non-PSC IBD. To reduce the bias in the correlation analysis due to non-normality, we removed the variables with more than eight null value results, and removed the measurements beyond the 5% quantile of the distribution. We computed the raw probabilities. The *p*-values of the Pearson's correlation were calculated using the corr.test function in R software with false discovery rate (FDR) adjustment for multiple comparisons. Spearman's correlations of the selected pairs with significant *p*-values in the Pearson correlations were also computed to check the consistency of the correlations. We listed the genus-BA pairs with both significant Pearson's correlation (adjusted $p < 0.05$) and strong Spearman's correlations ($p < 0.05$) in the Supplementary Material, Table 2.

Results

Study population

Thirty patients with IBD, of whom 15 had concomitant PSC, were prospectively enrolled. All patients enrolled had pancolitis; two out of the four patients with CD also had ileal involvement. Two of the 15 PSC patients had concomitant liver cirrhosis (Child-Pugh A, six points). No patient had a prior history of abdominal or liver surgery. There were no significant differences in the overall daily intake of macro or micronutrients as assessed by the food-frequency questionnaire (data not shown). Patients with PSC-IBD presented, as expected, significantly higher levels of cholestasis markers, and were more frequently medicated with ursodeoxycholic acid. No further significantly different clinical variables were found, except for body mass index (BMI) that was significantly lower in PSC-IBD patients (Table 1). The median interval between stool collection and colonoscopy was 17.5 days (9–62). The additional demographic and clinical characteristics of PSC and IBD patients are described in Table 1.

Serum BA

The total BA (μmol/l) pool was significantly expanded in PSC-IBD (*p*-value = 0.007, Mann–Whitney) (Supplementary Material, Table 1). No significant differences were seen in the proportion of individual BAs between groups. There was a positive correlation between PSC duration and total serum BAs ($\rho = 0.66$, $p = 0.009$).

Stool BA profiles

The median total stool BAs were significantly reduced in PSC-IBD (167.2 μmol/l in PSC-IBD versus 282.4 μmol/l in IBD, $p = 0.021$). Overall there were no significant differences in the proportions of each BA (Table 2), although the overall combination of stool BA allowed a good separation between PSC-IBD and IBD, as visualized in the linear discriminant analysis (Figure 1). Using the main BAs (CA, CDCA, LCA, DCA and UDCA), the classification accuracy of the LDA was 73%, with a sensitivity and specificity of 86.7% and 60% respectively (Figure 1). When we used all individual BAs (taurine and glycine conjugates and sulphated BA), the accuracy of the LDA for classifying PSC-IBD versus IBD was 100% (Supplementary Material, Figure 1). Additional LDA analysis was conducted using the top four most discriminatory stool BA (Supplementary Material, Figure 1). PSC-IBD patients presented a higher proportion of conjugated BA, although this did not reach statistical significance. DCA, a secondary BA, was also elevated, albeit non-significantly, in PSC-IBD. The proportion of UDCA in stool was not different in the PSC patients who were medicated with UDCA versus those who were not (1.075 nmol/g versus 1.35 nmol/g, respectively, *p*-value=0.7, Mann–Whitney U Test).

Table 1. Demographic and clinical characteristic of patients.

	PSC-IBD ($n=15$)	IBD ($n=15$)	p value[a]
Male (n, %)	5 (33%)	10 (67%)	0.07
Age (years)			
Median, IQR	42(24)	45 (13)	0.6
Smoking status (n, %)			
Never	12 (80%)	12 (80%)	1.0
Ever	3 (20%)	3 (20%)	
Type of IBD (n, %)			
UC	11 (73%)	12 (80%)	0.6
CD	4 (27%)	3 (20%)	
PSC duration			
Median years (IQR)	7.8 (11.7)	–	–
IBD duration			
Median years, (IQR)	11.4 (5.26)	11.1 (15.7)	0.8
PSC Mayo score			
Median, (min, max)	−0.57 (−1.6, 1.7)	–	–
ALP (UI/l) (median, IQR)	200 (166)	54 (28)	<0.001
GGT (UI/l) (median, IQR)	332 (414)	28 (20)	<0.001
CRP (mg/dl) (median, IQR)	0.2 (0.7)	1.1 (1.3)	0.061
Disease clinical activity (n, %)			
Remission-mild	13 (87%)	15 (100%)	0.5
Moderate-severe	2 (13%)	0	
Disease endoscopic activity (n, %)[b]			
Remission-mild	9 (64%)	13 (87%)	0.2
Moderate-severe	5 (36%)	2 (13%)	
Mean BMI (Kg/m^2)	24 ± 4.5	30.1 ± 6.4	0.005
Presence of colorectal dysplasia (n, %)	3/14 (21%)	1/15 (6%)	0.2
Medications (n, %)			
5-ASA	12 (80%)	11 (73%)	1.0
Thiopurines	5 (33%)	8 (53%)	0.3
Anti-TNF	3 (20%)	3 (20%)	1.0
UDCA	10 (67%)	–	<0.001

5-ASA: 5-aminosalicilate; ALP: alkaline phosphatase; anti-TNF: anti-tumor necrosis factor; BMI: body mass index; CRP: C-reactive protein; GGT: gamma-glutamyl transpeptidase; IQR: interquartile range; PSC: primary sclerosing cholangitis; PSC-IBD: primary sclerosing cholangitis associated with inflammatory bowel disease; UC: ulcerative colitis; UDCA: ursodeoxycholic acid.
[a]Variable distribution was compared using the Student's t test, the Mann–Whitney test or the χ^2 test, as appropriate; [b]in the PSC-IBD group one patient refused colonoscopy.

Likewise, the results for all stool BA comparisons did not change after excluding the two CD patients with ileal involvement (data not shown). There was a negative correlation between the concentration of secondary BAs and endoscopic disease activity ($\rho = -539$, $p = 0.003$); this was also observed when the analysis was stratified by patient group (data not shown).

Survey of gut microbiota

Using 16S rRNA sequencing, we surveyed the microbiome composition of 30 stool samples. The duplicate measurements showed Pearson correlation over 99% at genus level, confirming the reproducibility of the experimental approach. PSC-IBD presented lower alpha-diversity, albeit not significantly different (Chao1 899.3 for IBD vs 832.0 for PSC-IBD, p-value $= 0.36$; Shannon index 5.7 for IBD vs 5.3 for PSC-IBD, $p = 0.23$) (Figure 2(b)). Patients with PSC and concomitant cirrhosis ($n = 2$) presented significantly lower bacterial alpha-diversity ($p = 0.005$) as compared with those with PSC without cirrhosis (data not shown). The overall microbiota dissimilarities among all samples grouped by PSC and IBD status were accessed using the

UniFrac distance matrices (Figure 2(a)). The overall qualitative microbial composition of patients with PSC-IBD was different as observed in the multidimensional scaling (MDS) plot (Figure 2(a): unweighted UniFrac, PERMANOVA: pseudo-F statistic: 2.99, p value=0.008). At the individual taxa level (Figure 2(c)), we found seven genera differentially expressed in PSC-IBD vs IBD (logarithmic LDA score>2 by LEfSe analysis): *Ruminococcus* and *Fusobacterium* were more abundant in PSC-IBD, while *Dorea*, *Veillonella*, *Lachnospira*, *Blautia*, and *Roseburia* were less abundant. All of those genera were found to be significant ($p < 0.05$) when their relative abundance was compared using a Kruskal-Wallis test (p values adjusted for multiple comparisons). No significant differences in the microbial overall composition (ß-diversity) were observed by PSC disease severity (as measured by the PSC Mayo score), BMI, UDCA use, IBD type, or IBD disease activity (data not shown).

Table 2. Stool bile acids (BAs) in primary sclerosing cholangitis associated with inflammatory bowel disease (PSC-IBD) and inflammatory bowel disease (IBD) patients.

BAs	PSC-IBD	IBD	p value
Primary BAs	9.5 (18)	4.2 (15.2)	0.29
Secondary BAs	89.4 (24.6)	91.2 (15.1)	0.57
CA	4.6 (6.45)	1.49 (4.77)	0.06
CDCA	4.73 (10.4)	2.72 (11.0)	1.0
DCA	52.5 (23.5)	43.6 (14.3)	0.55
LCA	34.1 (33.8)	46.2 (1.9)	0.14
UDCA	1.1 (1.9)	1.8 (3.9)	0.37
Tauro/glyco conjugates	0.47 (0.67)	0.34 (0.69)	0.98
Sulfated BAs	2.1 (3.1)	2.4 (16.3)	0.41
Conjugated BAs	4.5 (13.7)	2.7 (6.9)	0.23

CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: litocholic acid; UDCA: ursodeoxycholic acid.
BAs are expressed as percentage median (interquartile range) of total BAs. Distributions were compared with non-parametric tests (Mann–Whitney). Due to a small amount of minor BA species in stool (muricholic acid, hycholic acid, hyodeoxycholic acid, or ursodeoxhycolic acid) which are considered by some authors as 'tertiary BAs', the sum of primary and secondary BAs is not 100%.

Differential OTUs by PSC status

Based on 97% similarity of the 16S sequencing reads, the open-reference OTU picking using QIIME pipeline assigned all sequencing reads into individual OTUs. After removing singletons, we compared 3839 OTUs and selected 143 OTUs which were significantly ($p < 0.05$ by Kruskal–Wallis test, not adjusted) differential and presented a >2 fold changes in the mean abundance between IBD and PSC-IBD (Supplementary Material, Figure 2). Compared with IBD only, the relative abundance of 32 OTUs were increased and 111 OTUs were decreased in PSC-IBD. At the phylum level, we found that most of the shifts associated with PSC occurred within the *Firmicutes* (73%) and *Bacteroidetes* phyla (17%). Consistent with the LEfSe analysis at the genus level, that found *Blautia* and *Ruminococcus* as the two most significant

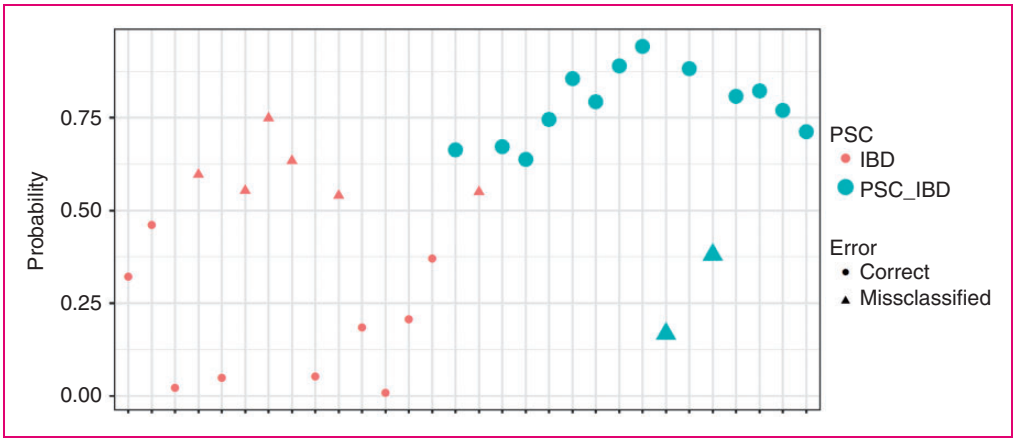

Figure 1. Results of the linear discriminant analysis allowing to see the discrimination of primary sclerosing cholangitis associated with inflammatory bowel disease (PSC-IBD) versus inflammatory bowel disease (IBD) alone, based on the combination of the main bile acids (BAs) present in stool (cholic acid (CA), chenodeoxycholic acid (CDCA), litocholic acid (LCA), deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA)). On the x axis each marker represents a patient. On the y axis is represented the probability of being correctly classified as PSC-IBD using the BA analytes. The green markers represent patients with PSC-IBD and the pink markers represent patients with IBD. The circles represents patients that were correctly assigned to their disease group. The classification accuracy of the linear discriminant analysis (LDA) was 73%, with a sensitivity and specificity of 86.7% and 60% respectively.

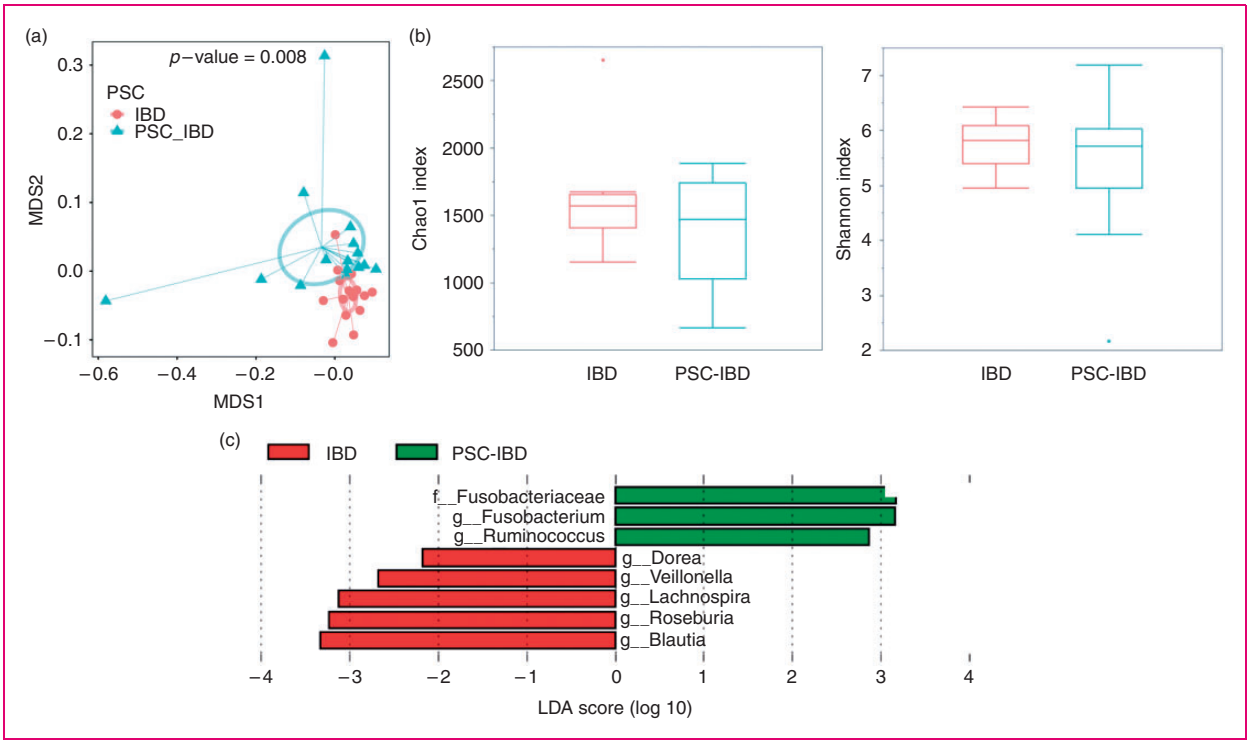

Figure 2. Overall microbiota dissimilarities between samples grouped by primary sclerosing cholangitis (PSC) and inflammatory bowel disease (IBD) status. (a) Dissimilarities were measured using UniFrac unweighted distances and visualized using a multidimensional plot (MDS) plot. The smaller circle represents patients with IBD, while the larger circle the samples from PSC-IBD patients. (b) The boxplots show the mean and variance of the richness of the microbial community between different disease status (Chao1 in the left and Shannon index on the right); no significant differences are seen (*p* value: 0.36 and 0.23 respectively). (c) Top discriminative bacteria in primary sclerosing cholangitis associated with inflammatory bowel disease (PSC-IBD) and IBD patients as determined by LEfSe analysis (linear discriminant analysis (LDA)). On the right are represented the increased taxa in PSC-IBD, while on the left the decreased taxa in PSC-IBD, as compared with IBD.

differential genera by PSC status, we found that all 16 OTUs of *Blautia* genus were reduced while four of five OTUs of *Ruminococcus* genus and *Ruminococcaceae* family were enriched in PSC samples.

Correlation between microbiota genera and stool BAs

Correlations between microbiota genus and the stool BA were calculated as described in our method section to test the interactions between gut microbiota and stool BA. Without stratifying by PSC status, we found four genera, including *Blautia* and *Veillonella* to be correlated to specific types of BAs. In PSC-IBD, bacteria with significant correlations with BA metabolites mostly belonged to the *Firmicutes* phylum, specifically within the *Clostridia* and *Bacilli* classes. Different correlations were observed in IBD (Supplementary Material, Table 2). Compared with IBD, seven genera appeared and two genera disappeared in PSC-IBD. The total relative abundance of genera correlated to BA was 12% in PSC-IBD, compared with 0.4% in IBD. Two *Firmicutes*, *Lachnospira* and *Veillonella*, which were significantly reduced in PSC-IBD, showed strong correlations with multiple BA, only in PSC-IBD.

Discussion

Herein, for the first time we have analyzed the stool BA profiles and their correlation with the faecal microbiota composition in patients with PSC-IBD as compared with IBD alone. The serum BA pool was increased and the stool BA pool was significantly reduced in PSC-IBD as compared with IBD alone. No significant differences in the individual stool BA components were found, but their overall composition differed from IBD (Figure 1). A significantly different microbiota composition based on the unweighted UniFrac distances was found between IBD and PSC-IBD, indicating differences in taxon composition for rare taxa (Figure 2(a)). Specifically, PSC-IBD patients presented an enrichment in bacteria belonging to the genera *Ruminococcus* and *Fusobacterium* as compared with IBD alone (Figure 2(c)). Finally, specific microbiota-stool BA correlations were observed in PSC-IBD (Supplementary Material, Table 2).

In the past, some authors have hypothesized that the increased risk of right-sided colorectal neoplasia in PSC-IBD could be linked to an increase in secondary BA, although this had never been demonstrated.[2,7] Normally, most of the BAs secreted by the liver are efficiently reabsorbed in the terminal ileum, through the sodium-dependent BA transporter (ASBT), leaving only approximately 5% of the total BAs to reach the colonic lumen. In the right colon, primary BAs are transformed in to secondary BAs mostly by bacterial mediated deconjugation, oxidation/reduction, epimerization, and dehydroxylation.[40] Therefore, faecal BA are mainly deconjugated, secondary BAs. A small fraction of secondary BA is passively absorbed through the colonic mucosa, whilst the rest will be extruded with faeces.[41] During obstructive cholestasis, the expression of the apical BA transporter, which permits intracellular absorption of BAs, is down-regulated, as a compensatory mechanism.[42] This could hypothetically lead to a relative increase in the proportion of BAs entering the proximal colon in PSC-IBD patients, where they would be converted from primary into secondary BAs. Interestingly, secondary BAs have been shown to have anti-inflammatory properties but at the same have been shown to bear carcinogenic properties.[19,32,43–45] Herein, we observed a significant reduction in the total stool BAs in PSC-IBD as compared with IBD, which was expected taking into consideration the obstructive cholestatic nature of PSC. However, we did not find an increase in the relative proportion of the stool secondary BAs in PSC-IBD patients, as previously hypothesized.[7,12] No significant differences in individual proportion of serum or stool BAs were found, which could perhaps be due to the small sample size. The proportion of DCA, a secondary BA was increased in PSC-IBD, although this did not reach statistical significance. Furthermore, the proportion of conjugate BAs was also non-significantly increased in PSC-IBD as compared with IBD, which could indirectly indicate a decrease in the deconjugation activity of the microbiota. The decrease in *Bacteroides*, *Clostridium*, *Bifidobacterium*, and *Lactobacillus* genus, observed at the OTU level, and known to be involved in BA deconjugation, could hypothetically be involved in this finding.[46] In this cohort, patients with PSC-IBD demonstrated an enrichment in bacteria from the *Ruminococcus* and *Fusobacterium* taxa, and a decrease in bacteria from the genus *Dorea*, *Veillonella*, *Lachnospira*, *Blautia*, and *Roseburia*. At the OTU level most shifts were observed within the *Firmicutes* (73%) and *Bacteroidetes* phylae (17%). Some of our findings are in consonance with recently published results on PSC microbiota also showing an increase of *Fusobacterium*[13] (a bacterial taxon that has been linked with adenomas and colorectal cancer) and in *Ruminococcus* in stool from patients with PSC-IBD or a decrease in *Roseburia* genus. However, others are not; Kummen et al. reported PSC patients to have a significant increase in *Veillonella* genus in comparison with healthy controls and patients with IBD.[14] In this cohort, *Veillonella* genus was positively correlated with disease severity and was more abundant in patients that had undergone OLT. Indeed, this genus has been reported to be increased in fibrotic conditions such as liver or lung fibrosis or cystic fibrosis.[47–50] In cirrhosis, *Veillonella* has also been associated with complications such as hepatic encephalopathy.[51] In another large cohort of patients with PSC-IBD, *Veillonella* was only significantly elevated in patients with PSC that presented concomitant liver cirrhosis.[13] Of note, in our PSC population, only two patients presented early liver cirrhosis, no patient presented severe PSC as measured by the PSC Mayo score or had undergone liver transplant. Also worthy of note is the well described disconnect between mucosa and stool microbiota, as in a prior work *Blautia* was increased in the mucosa from PSC-IBD patients as compared with healthy controls.[12]

No study had yet looked at the correlations between stool BA and the stool microbiota in PSC-IBD. However, BA pool size and composition have been shown to be important factors in regulating the gut microbiota.[24,52,53] Herein, despite our relatively small sample size, and after correcting for multiple comparisons, we were able to observe unique correlations between stool microbiota and stool BAs in PSC-IBD. Within IBD alone, this broad BA-microbiota correlation disappeared. In particular, in PSC-IBD, the taxa that significantly correlated to the stool BA corresponded to ~12% of the total microbiota, while in IBD, this was less than 1%. Without any functional data, we may only speculate that these results suggest that under PSC conditions, the BA changes may have dominant effects on defining the gut microbiota shifts, potentially towards a more pro-carcinogenic profile. Interestingly, bacteria from the genus of both *Fusobacterium* and *Ruminococcus*, are known to be involved in oxidation, epimerization and desulfation of BAs.[46]

The major limitation of this study is our small sample size, which prevented us from adjusting for potential confounders in the microbiota and BA analysis. To overcome this, we tried to make our cohort as uniform as possible. All patients had pancolitis, and no patient had prior abdominal surgery or history of liver transplantation; all patients had mild to moderate PSC, as measured by the Mayo score, and dietary intake was also similar within groups as assessed by the food frequency questionnaire. Furthermore, all patients stopped UDCA intake for two weeks and had no antibiotics or bowel preparation within at least three months of sample collection, all external factors that could potentially impact microbiota composition.

While it may be argued that a two-week interval to stop UDCA may not be enough to remove its effects, we did not observe any differences in the faecal BA composition or in the microbiota composition between those who were medicated with UDCA as compared with those who were not, consistent with what has been previously reported.[13]

In summary, in this exploratory study, patients with PSC-IBD had a distinct stool BA and stool microbiota composition, as well as specific microbiota-stool BA correlations when compared with IBD. Whether these changes are associated with or may predispose to the specific PSC-IBD phenotype including the increased risk of colorectal neoplasia needs to be further clarified and warrants further research.

Acknowledgements

The authors wish to thank the OCS genome technology center of New York University Langone Medical Center for the Illumina Miseq molecule library preparation and sequencing service. JT, SHI, MC, JFC, and JH have contributed to the study design and concept. JT, CP, MC, XB, PMS, JPS, CV, and AO have contributed to the acquisition of data. JT, RH, KP, HB, CR, DR, LH and JH analyzed, and interpreted the data. JT and JH drafted the manuscript. All authors have reviewed the final version of the manuscript and provided critical comments. JT and JH obtained funding support.

Declaration of conflicting interests

J Torres has received consulting fees from Takeda. J-F Colombel has served as consultant, advisory board member or speaker for Abbvie, AB Science, Amgen, Bristol Meyers Squibb, Celltrion, Danone, Ferring, Genentech, Giuliani SPA, Given Imaging, Janssen, Immune Pharmaceuticals, Medimmune, Merck & Co., Millenium Pharmaceuticals Inc., Neovacs, Nutrition Science Partners Ltd, Pfizer Inc., Prometheus Laboratories, Protagonist, Receptos, Sanofi, Schering Plough Corporation, Second Genome, Shire, Takeda, Teva Pharmaceuticals, Tigenix, UCB Pharma, Vertex, and Dr August Wolff GmbH & Co. SH Itzkowitz received grant support and fees for serving on a scientific advisory board from Exact Sciences. The remaining authors have no conflicts of interest to declare.

Ethics approval

This study was approved by the Portuguese National Committee for Data Protection and by the local ethics committee at each participating center.

Funding

This study was supported by funds from GEDII (Portuguese Group for the Study of Inflammatory Bowel Disease) (JT) and from the National Institute of Health (NIDDK 1K01DK094986-01(JH) and NIDDK R03 DK106481-01(JH)).

Informed consent

All patients participating in this study signed an informed consent form.

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HIGH RISK OF ADVANCED COLORECTAL NEOPLASIA AND ACCELERATED DYSPLASIA-CARCINOMA SEQUENCE IN PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

Clinical Gastroenterology and Hepatology 2018;■:■–■

High Risk of Advanced Colorectal Neoplasia in Patients With Primary Sclerosing Cholangitis Associated With Inflammatory Bowel Disease

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BACKGROUND & AIMS: **Patients with inflammatory bowel disease (IBD) and primary sclerosing cholangitis (PSC, termed PSC-IBD) are at increased risk for colorectal cancer, but their risk following a diagnosis of low-grade dysplasia (LGD) is not well described. We aimed to determine the rate of advanced colorectal neoplasia (aCRN), defined as high-grade dysplasia and/or colorectal cancer, following a diagnosis of indefinite dysplasia or LGD in this population.**

METHODS: **We performed a retrospective, longitudinal study of 1911 patients with colonic IBD (293 with PSC and 1618 without PSC) who underwent more than 2 surveillance colonoscopies from 2000 through 2015 in The Netherlands or the United States (9265 patient-years of follow-up evaluation). We collected data on clinical and demographic features of patients, as well as data from each surveillance colonoscopy and histologic report. For each surveillance colonoscopy, the severity of active inflammation was documented. The primary outcome was a diagnosis of aCRN during follow-up evaluation. We also investigated factors associated with aCRN in patients with or without a prior diagnosis of indefinite dysplasia or LGD.**

RESULTS: **Patients with PSC-IBD had a 2-fold higher risk of developing aCRN than patients with non-PSC IBD. Mean inflammation scores did not differ significantly between patients with PSC-IBD (0.55) vs patients with non-PSC IBD (0.56) ($P = .89$), nor did proportions of patients with LGD (21% of patients with PSC-IBD vs 18% of patients with non-PSC IBD) differ significantly ($P = .37$). However, the rate of aCRN following a diagnosis of LGD was significantly higher in patients with PSC-IBD (8.4 per 100 patient-years) than patients with non-PSC IBD (3.0 per 100 patient-years; $P = .01$). PSC (adjusted hazard ratio [aHR], 2.01; 95% CI, 1.09–3.71), increasing age (aHR 1.03; 95% CI, 1.01–1.05), and active inflammation (aHR, 2.39; 95% CI, 1.63–3.49) were independent risk factors for aCRN. Dysplasia was more often endoscopically invisible in patients with PSC-IBD than in patients with non-PSC IBD.**

[a]Authors share co-first authorship.

Abbreviations used in this paper: aCRN, advanced colorectal neoplasia; aHR, adjusted hazard ratio; CRC, colorectal cancer; EHR, electronic health record; HGD, high-grade dysplasia; IBD, inflammatory bowel disease; IBD-U, inflammatory bowel disease undifferentiated; IND, indefinite dysplasia; LGD, low-grade dysplasia; PSC, primary sclerosing cholangitis; UC, ulcerative colitis.

1542-3565/$36.00
https://doi.org/10.1016/j.cgh.2018.01.023

CONCLUSIONS: **In a longitudinal study of almost 2000 patients with colonic IBD, PSC remained a strong independent risk factor for aCRN. Once LGD is detected, aCRN develops at a higher rate in patients with PSC and is more often endoscopically invisible than in patients with only IBD. Our findings support recommendations for careful annual colonoscopic surveillance for patients with IBD and PSC, and consideration of colectomy once LGD is detected.**

Keywords: Surveillance; Colon Cancer; Crohn's Disease; Ulcerative Colitis; Primary Sclerosing Cholangitis.

Patients with inflammatory bowel disease (IBD) are at an increased risk of developing colorectal cancer (CRC).[1,2] The co-occurrence of primary sclerosing cholangitis (PSC),[1,3] a chronic liver disease characterized by progressive inflammation and fibrosis of the bile ducts,[4] increases this risk substantially.[5] Although an estimated 70% of patients with PSC have a concomitant diagnosis of IBD (termed PSC-IBD),[6] only 3% to 5% of patients with IBD have concomitant PSC, with the diagnosis more common in patients with ulcerative colitis (UC).[5,7] The PSC-IBD phenotype often is characterized by extensive colitis with rectal-sparing and backwash ileitis, albeit with a mild and often asymptomatic clinical course.[8–13] However, despite their mild clinical colitis, patients with PSC-IBD compared with patients with only IBD colitis have a 3- to 5-fold higher risk of CRC, and the cancers occur more often in the right colon.[14,15] As such, current guidelines recommend that patients with PSC-IBD be enrolled in a CRC surveillance program with an annual colonoscopy from the time of PSC diagnosis, regardless of their duration of IBD. This is in contrast to patients with IBD colitis and no PSC (non-PSC IBD), in which CRC surveillance is recommended after 8 years of colonic disease.[5,16–18]

The development of neoplasia in IBD colitis follows a multistep sequence from chronic inflammation and no dysplasia or indefinite dysplasia (IND) to low grade-dysplasia (LGD) and high-grade dysplasia (HGD), before final malignant transformation to adenocarcinoma. As such, the presence and grade of dysplasia remain the best current indicators of cancer risk in IBD. There is an increasing tendency to keep patients with LGD on intensive surveillance instead of recommending proctocolectomy.[19,20] However, very few studies have described the risk of advanced colorectal neoplasia (aCRN) in patients with PSC-IBD after a diagnosis of IND and/or LGD.[21,22] Furthermore, the studies that do report on the risk of neoplasia in patients with PSC-IBD were performed in an era in which imaging-enhanced endoscopy and high-resolution endoscopy were not used routinely.

The aims of the present study were to report on the risk of aCRN in a well-characterized cohort of patients with PSC-IBD enrolled in a surveillance program in the modern endoscopic era, and to describe the rate of aCRN after a diagnosis of IND and/or LGD in these patients compared with patients with non-PSC IBD and long-standing IBD colitis also undergoing surveillance.

Methods

Study Population and Case Identification

Patients with established IBD colitis undergoing colonoscopic surveillance between 2000 and 2015 were identified retrospectively from 2 databases: a Dutch database inclusive of 2 secondary and 6 tertiary centers and the Mount Sinai Hospital database in New York City inclusive of 1 tertiary IBD referral center. Cases were identified by query of the electronic health record (EHR)-linked database using both International Classification of Diseases, 9th and 10th revision codes, and free text searches for cases of IBD and also free text searches for PSC.

Patient Selection: Inclusion and Exclusion Criteria

After initial identification through the EHR query, individual charts were reviewed. For patients with PSC-IBD, a clinical diagnosis of PSC had to be confirmed by distinctive features on cholangiography or liver biopsy (for patients with small-duct PSC). Additional inclusion criteria were as follows: (1) diagnosis of IBD (UC, CD, IBD undifferentiated [IBD-U]) with colonic involvement confirmed endoscopically and histologically; (2) confirmed colonic disease duration of at least 8 years for patients with non-PSC IBD or any colonic disease duration for patients with PSC-IBD; (3) enrollment in a surveillance program; and (4) at least left-sided colitis (UC or IBD-U) or involvement of more than 30% of the colonic surface (CD or IBD-U). Patients with a history of colectomy before enrollment or a history of aCRN before or at the index colonoscopy during the defined study period were excluded. Surveillance procedures were defined as colonoscopies in which either segmental random biopsies or chromoendoscopy were used. Colonoscopies with other indications (eg, medically refractory disease), were excluded. The index colonoscopy was defined as the first surveillance colonoscopy performed within the study period (2000–2015).

Data Collection

Database coding was identical for all study populations. The date of study entry was set at the first surveillance colonoscopy in the database. The time of

onset of PSC or IBD was determined from EHR review. The date of the last colonoscopy was set as the last day of follow-up evaluation.

The following baseline demographic and clinical data were abstracted: date of birth, sex, date of PSC diagnosis (if applicable), date of IBD diagnosis, IBD type, maximum disease extent, and date of prior diagnosis of IND and/or LGD (if applicable). Maximum disease extent was defined as the maximum documented extent of endoscopic disease on any colonoscopy and was coded as follows: extensive/pancolitis (>50%) or intermediate/left-sided (30%–50%). Medication exposure (at least 1 prescription) was recorded for mesalamine, thiopurines, and biologics.

Data from each surveillance colonoscopy were recorded, including date of examination, quality of bowel preparation (adequate or inadequate), most proximal extent examined, use of chromoendoscopy, presence and severity of endoscopic inflammation, presence of post-inflammatory polyps (pseudopolyps), stricture(s), and visible lesions. Endoscopically detected neoplastic lesions were categorized based on morphology (polypoid/nonpolypoid). Endoscopically invisible neoplasia was defined as neoplasia detected in a random biopsy with no corresponding morphologic lesion seen on endoscopy. Right-sided lesions were defined as those proximal to the splenic flexure. Because this was a retrospective study, there was no a priori protocol in place to record endoscopic activity in a uniform way. Thus, for each surveillance colonoscopy, the severity of active endoscopic inflammation was scored on a 4-point scale for each colonic segment visualized to allow for standardization: 0, no inflammation/remission; 1, mild inflammation; 2, moderate inflammation; or 3, severe inflammation. A mean inflammatory severity score per patient and per colonoscopy was calculated by dividing the sum of inflammatory severity scores by the total number of colonic segments visualized per colonoscopy and then by the total number of surveillance colonoscopies.

Histology

Dysplasia was recorded as IND, LGD, or HGD. All histologic diagnoses were as reported in the original pathology report; no specimens were re-reviewed or altered for this study. Of note, it is routine clinical practice at each participating institution that colorectal neoplasia is reviewed at the time of diagnosis and agreed upon by at least 2 pathologists.

Primary and Secondary Outcomes

The primary outcome was a diagnosis of aCRN, defined as HGD or CRC, during follow-up evaluation. Secondary outcomes were a diagnosis of IND and/or LGD during follow-up evaluation and the development of aCRN after a diagnosis of IND and/or LGD. Factors associated with a diagnosis of aCRN in patients with PSC-IBD or patients with non-PSC IBD with or without a prior diagnosis of IND and/or LGD were explored.

Statistical Analysis

Basic descriptive statistics were generated for patients meeting inclusion criteria. Chi-square and Fisher exact tests were used to compare categoric variables and dichotomous outcomes, whereas the Student *t* test and the Mann–Whitney *U* test were used for analyzing continuous data. Incidence rates were calculated as the number of cases per 100 patient-years of follow-up evaluation. Univariate and multivariate Cox-regression modeling was used to identify factors associated with aCRN. The proportional hazards assumption of time-static covariates was assessed using log–log plots and Schoenfeld residuals. Because inflammatory scores were not stable over time, these were input as time-changing covariates into the models. Mean inflammation scores were recalculated at every time point for each patient to correct for the variable number of colonoscopies. A P value of .10 or less was used as the cut-off value for selecting variables for the multivariate analysis. Kaplan–Meier survival curves were generated to compare cumulative incidence rates. Follow-up data were censored at the last point of colonoscopic follow-up evaluation, aCRN diagnosis, or colectomy. All data analyses were performed using SPSS version 22 (IBM Corp, Armonk, NY).

Study Oversight

The Institutional Review Board at each of the included sites approved the creation and analysis of a longitudinal retrospective cohort database of patients with colonic IBD undergoing surveillance.

Results

Baseline Demographic and Clinical Characteristics

Of 1911 patients with colonic IBD in the combined database meeting inclusion criteria, 293 patients were confirmed to have PSC-IBD; the remaining 1618 patients with non-PSC IBD served as the comparison group (Figure 1). The main demographic and clinical features of the cohort are detailed in Table 1. Compared with the non-PSC IBD group, patients with PSC-IBD were more often male and younger at study entry, although the age at IBD diagnosis was similar between groups ($P = .11$). As expected, UC was the predominant IBD type in the PSC-IBD group. Patients with PSC-IBD were less frequently exposed to IBD therapy compared with patients with non-PSC IBD. In 151 patients (51.5%), the PSC diagnosis was established after the IBD diagnosis, while in 36 patients (12.3%) PSC was established before the IBD diagnosis. For the remainder, PSC and IBD were

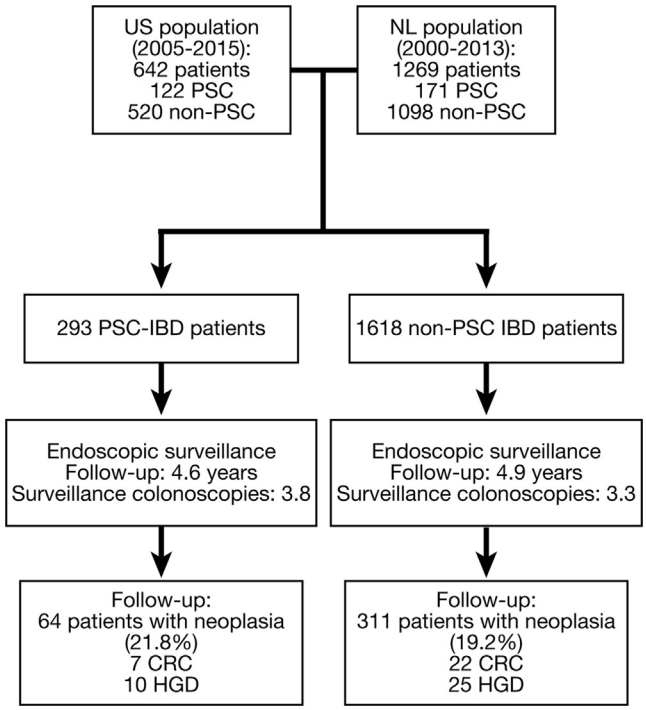

Figure 1. Description of patient selection and main outcomes in each database. NL, The Netherlands.

Table 1. Baseline Characteristics of the Study Population

	PSC-IBD (n = 293)	Non-PSC IBD (n = 1618)	*P* value
Male, %	205 (70.0%)	796 (49.2%)	<.001
Age at study inclusion, *y*, means (SD)	39 (14)	45 (13)	<.001
IBD type			<.001
Ulcerative colitis	203 (69.3%)	912 (56.4%)	
Crohn's colitis	76 (25.9%)	661 (40.9%)	
Indeterminate colitis	14 (4.8%)	45 (2.8%)	
Disease extent			<.001
Not specified	34 (11.8%)	154 (9.6%)	
Limited extent/proctitis	13 (4.5%)	49 (3.1%)	
Intermediate/left-sided	41 (14.2%)	572 (35.8%)	
Extensive/pancolitis	201 (69.6%)	823 (51.5%)	
Age at IBD diagnosis, *y*, mean (SD)	27 (13)	28 (12)	.11
IBD duration, *y*, mean (SD)	12 (10)	17 (9)	<.001
Age at PSC diagnosis, *y*, mean (SD)	32 (14)	-	-
Medication use			
Mesalamine	221 (75.4%)	1316 (81.3%)	.02
Thiopurines	93 (31.7%)	825 (51.0%)	<.001
Biologicals	38 (13.0%)	402 (24.8%)	<.001
Duration of follow-up evaluation after index colonoscopy, *y*			.10
Means (SD)	4.6 (3.2)	4.9 (3.0)	
Median	4.1	4.5	
Number of surveillance colonoscopies, mean	3.8	3.3	<.001

diagnosed within the same year or the sequence of diagnoses was not recorded.

The mean follow-up period for the total cohort was 4.8 years (SD, ±3.0 y), with a total of 9265 patient-years of follow-up evaluation; there was no difference in follow-up time between patients with PSC-IBD and patients with non-PSC IBD. The number of surveillance colonoscopies performed within the study period was higher in patients with PSC-IBD compared with patients with non-PSC IBD (3.8 vs 3.3; $P < .01$).

Inflammatory Activity

The endoscopic severity of inflammation on surveillance examinations was similar between patients with PSC-IBD and patients with non-PSC IBD (Supplementary Table 1). The proportion of procedures in which extensive active disease was observed in patients with PSC-IBD vs patients with non-PSC IBD patients was 27% vs 12% ($P < .01$), 23% vs 10% ($P < .01$), and 27% vs 10% ($P < .01$) for the first, second, and third surveillance colonoscopy, respectively. The proportion of patients in endoscopic remission on each of their surveillance colonoscopies during the entire study period was higher in patients with non-PSC IBD compared with patients with PSC-IBD ($P = .02$).

Occurrence of Advanced Colorectal Neoplasia and Associated Risk Factors

Among patients with PSC-IBD, aCRN was diagnosed in 17 patients (5.8%), with CRC in 7 (2.4%) and HGD in 10 patients (3.4%) (Table 2). The frequency of aCRN during follow-up evaluation was significantly lower in patients with non-PSC IBD (2.9%), with CRC and HGD diagnosed in 1.4% and 1.5% patients, respectively ($P = .01$). The incidence rate of aCRN in patients with PSC-IBD compared with patients with non-PSC IBD was significantly higher (1.3 vs 0.6/100 patient-years; $P < .01$) (Figure 2). Although aCRN was more often right-sided in patients with PSC-IBD compared with patients with non-PSC IBD, this was not statistically significant (53% vs 31%; $P = .12$). Among 40 patients with PSC-IBD (14%) in whom the diagnosis of PSC was newly established within the study period, 3 cases of aCRN occurred, with a mean duration of 4.0 years (±2.5 y) between the PSC diagnosis and aCRN occurrence. The primary outcomes stratified by study site are detailed in Supplementary Table 2.

On multivariate Cox-regression analysis, PSC (adjusted HR [aHR], 2.01; 95% CI, 1.09–3.71), increasing age (aHR, 1.03; 95% CI, 1.01–1.05), and active inflammation (aHR, 2.39; 95% CI, 1.63–3.49) remained independent predictors of aCRN diagnosis during follow-up evaluation (Table 3). Correcting for geography (United States vs The Netherlands) did not affect these findings (Supplementary Table 3).

Table 2. Description of the Outcomes During the Study Period

	PSC-IBD (n = 293)	Non-PSC IBD (n = 1618)	*P* value
Advanced neoplasia (aCRN)	17 (5.8%)	47 (2.9%)	.01
CRC	7 (2.4%)	22 (1.4%)	.19
HGD	10 (3.4%)	25 (1.5%)	.03
LGD, patients with ≥1 LGD lesion	60 (20.5%)	295 (18.2%)	.37
IND, patients with IND as highest grade lesion	27 (9.2%)	74 (4.6%)	.001
Time from IBD diagnosis to aCRN diagnosis, *y*, mean	19.4	24.3	.15
Time from database entry to aCRN diagnosis, *y*, mean	4.2	3.4	.31
Time from LGD to aCRN diagnosis, *y*, mean	0.7	1.7	.12

Risk of Advanced Colorectal Neoplasia After a Diagnosis of Indeterminate Dysplasia and/or Low-Grade Dysplasia

The number of patients in the total cohort with at least 1 diagnosis of IND was 147 (7.7%). In 101 patients (5.3%) no additional dysplasia was detected. Among patients with a diagnosis of IND, the rate of developing aCRN after detection of IND was higher in patients with PSC-IBD compared with patients with non-PSC IBD ($P = .02$) (Supplementary Figure 1). However, when patients with a synchronous or metachronous diagnosis of LGD (n = 46) were excluded from this analysis (ie, no grade of dysplasia higher than IND), this difference was no longer significant.

The occurrence of at least 1 LGD-containing lesion during the study period was similar for both patients with PSC-IBD and patients with non-PSC IBD (21% vs 18%; $P = .37$). Despite a similar proportion of patients with LGD, the rate of developing aCRN after detection of LGD was almost 3-fold higher in patients with PSC-IBD compared with patients with non-PSC IBD (8.4 vs 3.0/100 patient-years; $P = .01$) (Figure 3).

For the subgroup of patients with LGD, the number of patients in whom endoscopically invisible LGD was detected over the course of surveillance was higher in patients with PSC-IBD (38% vs 22%; $P = .01$). The proportion of invisible LGD cases among the total number of LGD cases (per-colonoscopy analysis) also was higher. In a subanalysis of The Netherlands population, we corrected for the total number of random biopsy specimens taken (107,745 biopsy specimens in total); the number of random biopsy specimens needed to detect invisible dysplasia was 826 in patients with PSC-IBD compared with 1703 in patients with non-PSC IBD.

On univariate Cox regression analysis, only PSC and multifocal dysplasia were associated with a higher risk of aCRN diagnosis after LGD detection, whereas polypoid morphology of the lesion (vs nonpolypoid or invisible) was associated with a lower risk. On multivariate analysis, only polypoid morphology remained significant and was associated with a reduced risk of aCRN (aHR, 0.31; 95% CI, 0.14–0.65) after LGD detection compared with nonpolypoid or endoscopically invisible lesions (Supplementary Table 4).

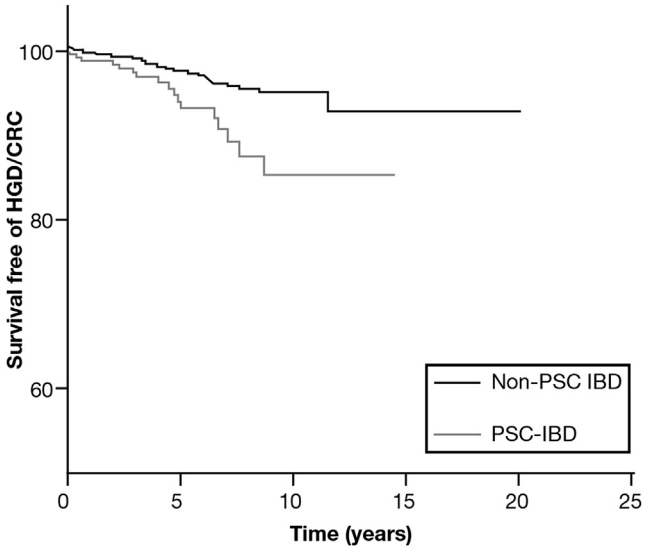

Figure 2. Kaplan–Meier time-to-event (aCRN) analysis, all patients since study entry.

Discussion

In this large, multicenter, cross-national, longitudinal cohort study of patients with confirmed IBD colitis undergoing colonoscopic CRC surveillance, we report a higher risk of aCRN in patients with concomitant PSC compared with those without PSC, in the current era of improved endoscopic technology and more effective medical therapy for inflammation. Although these findings corroborate previous studies, we further expand knowledge in the field by reporting an even higher risk of aCRN after detection of LGD (but not IND alone). That LGD was endoscopically invisible more often in patients with PSC-IBD compared with patients with non-PSC IBD justifies the more intensive management considerations for this population. Our findings suggest that although continued meticulous CRC surveillance with annual colonoscopy is indicated in the absence of dysplasia for patients with PSC-IBD, the detection of LGD or higher-grade pathology should lead to a careful weighting of the pros and cons of more aggressive therapeutic management, including colectomy.

In our well-characterized surveillance cohort of patients with PSC-IBD undergoing surveillance, we found

Table 3. Univariate and Multivariate Cox-Regression Analysis for the Overall Risk of aCRN: All Patients

Variable	Univariate			Multivariate		
	HR	95% CI	*P* value	aHR	95% CI	*P* value
Age, *y*	1.02	1.01–1.04	.03	1.03	1.01–1.05	.007
Age at IBD diagnosis	1.00	0.98–1.02	.78			
Sex, reference: male	1.83	1.08–3.08	.02	1.62	1.94–2.79	.08
PSC	2.13	1.22–3.70	.01	2.01	1.09–3.71	.03
Inflammation severity, mean (0–3)[a]	2.14	1.48–3.09	<.001	2.39	1.63–3.49	<.001
IBD type, reference: UC	0.99	0.60–1.61	.95			
Maximum disease extent, reference: pancolitis	1.43	0.85–2.41	.18			
Thiopurine exposure	0.84	0.51–1.40	.85			
Biological exposure	0.72	0.36–1.46	.36			
Mesalamine exposure	1.14	0.58–2.25	.70			
Number of surveillance procedures	0.96	0.84–1.09	.53			

[a]Entered as time-changing covariate; 0, no inflammation/remission; 1, mild; 2, moderate; 3, severe.

that having PSC-IBD is associated with a 2-fold higher risk of aCRN. This risk is slightly lower than prior studies and a recent meta-analysis of 16 studies that reported a 3.4-fold higher odds for colorectal neoplasia in patients with PSC-IBD.[23] Importantly, the increased risk in our study remained after correcting for active endoscopic inflammation over time (which was used as a time-changing covariate rather than a mean overall score). Endoscopic activity assessed during subsequent colonoscopies was associated strongly with the risk of future aCRN, congruent with studies in the population with non-PSC IBD.[24–26] Whether the outcomes of endoscopic inflammation compared with histologic inflammation are distinct remains a question for future investigation.

The increased risk of CRC in patients with PSC and concomitant colonic IBD has firmly been established, although the underlying mechanisms remain unclear.[3,27–30] The nearly 3-fold higher rate of aCRN (HGD and/or CRC) diagnosis after LGD detection, as well as the difference in location, morphology, and endoscopic conspicuousness of dysplasia in patients with PSC-IBD compared with patients with non-PSC IBD, suggests nuances in the pathogenesis of neoplasia between these groups. Several investigators have proposed a role for altered colonic bile composition in carcinogenesis. A right-sided predominance of neoplasia reinforces this hypothesis, as well as several studies directly measuring the bile acid composition in both animals and human beings.[29,31,32] There is also evidence supporting the notion that patients with PSC have an altered colonic microbiome irrespective of concurrent IBD or ursodeoxycholic acid treatment.[33] Still, whether these bacterial alterations are a cause or a consequence of the disease characteristics specific to PSC remains to be clarified. Patients with PSC also share a distinct genotype,[34] which may predispose them further to neoplastic progression. More likely, the underlying etiologies are multifactorial with roles for gene–environment interactions, the microbiome, and epigenetic modifications. Further investigations will hopefully open new avenues for novel therapeutic discovery and primary and secondary prevention.

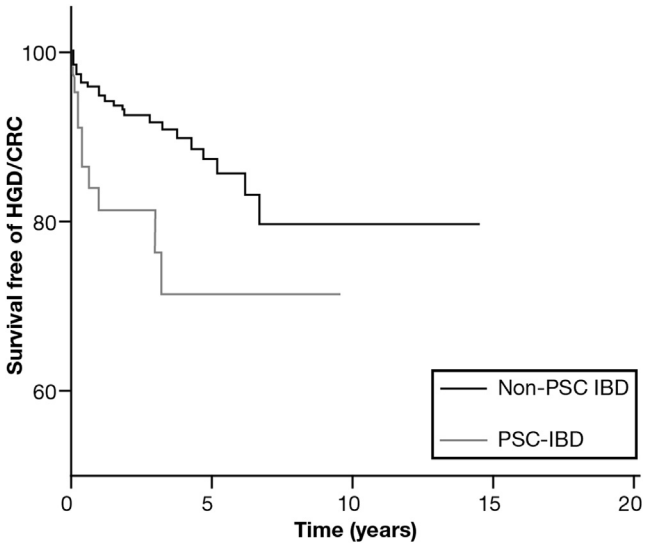

Figure 3. Kaplan–Meier time-to-event (aCRN) analysis, patients with LGD only.

All told, because the mechanisms underlying PSC as an independent risk factor for CRC in the setting of IBD colitis are unclear, the best strategy for CRC prevention in patients with PSC-IBD remains frequent, attentive surveillance colonoscopy. An important observation from our study that distinguishes patients with PSC-IBD from patients with non-PSC IBD, is that dysplasia was detected more often in random biopsies. Although previous retrospective studies have shown a low overall yield for dysplasia with random biopsies as opposed to only targeted biopsies of visible lesions, there was higher yield for dysplasia on random biopsy in patients with concurrent PSC.[35,36] Our data further add to this body of evidence, and it therefore can be questioned whether the current recommendation, based on the results of prospective studies, to move away from random biopsies as part of CRC surveillance should apply to patients with PSC-IBD.[37,38] During surveillance examinations, particular attention should be paid to the proximal colon because right-sided cancers seem to be more common in patients

with PSC-IBD compared with patients with non-PSC IBD colitis.[32] Although the proportion of right-sided aCRN was higher in the PSC-IBD subgroup, this difference was not statistically significant in the present study and may be owing to insufficient power; it also may reflect selection bias because one of our inclusion criteria for the non-PSC IBD subgroup was at least left-sided disease extent or more than 30% involvement, and thus may not represent the overall IBD population. Our study confirms that the date of PSC diagnosis is particularly relevant when risk-stratifying patients because it seems that the risk of neoplastic progression is highest within the first few years of the PSC diagnosis.[39] Thus, although CRC surveillance is recommended after a disease duration of 8 years in patients with colonic IBD and no PSC,[40] CRC surveillance at the time of diagnosis in the setting of PSC is recommended and further corroborated by our findings.

Our study had several strengths. In addition to being a large IBD surveillance cohort in the modern era, our cohort is particularly robust because each patient was confirmed to have colonic IBD and to be actively enrolled in a colonoscopic CRC surveillance program. Comprehensive data on disease history and endoscopic findings during surveillance allowed for more accurate neoplastic risk assessment, particularly with respect to measurement of inflammatory burden over time. Importantly, detailed information on inflammatory activity at each colonoscopy was incorporated into the analysis for more accurate assessment of aCRN development in patients with PSC-IBD.

Our study also had some limitations, most notably the retrospective design. Despite the large size of our PSC-IBD cohort, additional subanalyses, such as stratification according to IBD type or medication use, yielded insufficient power to permit meaningful conclusions. Although we combined surveillance cohorts from 2 different countries, we predefined the inclusion/exclusion criteria, variables to be assessed, and definitions of outcomes. Combining these 2 cohorts enhanced not only our power to detect meaningful differences, but also the generalizability of our findings given that our study population included patients from affiliated community-based sites as well as tertiary IBD referral centers. That said, there may be unmeasured differences in care pathways between The Netherlands and the United States, leading to heterogeneity in our study results. It is important to note, however, that after adjusting for study site and clinical–demographic differences between The Netherlands and US cohorts, our results remained significant (Supplementary Table 3). The lack of standardized guidelines for the use of chromoendoscopy for CRC surveillance in patients with IBD colitis unfortunately precluded a meaningful analysis of its impact on dysplasia detection because 10% or fewer examinations were performed with chromoendoscopy. Finally, although no samples were re-reviewed by pathologists for the purposes of this study, it is routine practice at all institutions participating in this study that whenever there is a diagnosis of CRN, that the specimen is reviewed by 2 pathologists and consensus is reached before final reporting.

In summary, using a large well-characterized cohort of patients with confirmed colonic IBD undergoing surveillance between 2000 and 2015, we substantiated prior smaller reports of the increased risk of aCRN in patients with concurrent PSC-IBD compared with patients with only IBD colitis undergoing surveillance. Novel findings of our study include the significantly higher rate of aCRN diagnosis after a diagnosis of LGD in the setting of PSC complicating IBD. This finding together with a higher risk of invisible dysplasia in patients with PSC-IBD highlights the need for an ongoing strict CRC surveillance program in these patients and a low threshold to advise colectomy once LGD is detected in this select population.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2018.01.023.

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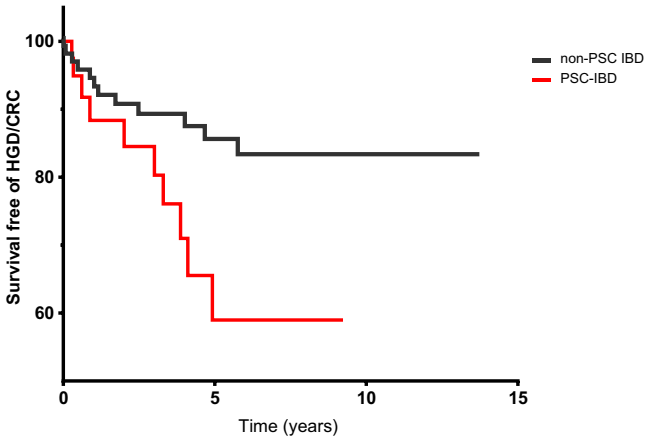
Conflicts of interest
The authors disclose the following: J.–F.C. received research grants from AbbVie, Janssen Pharmaceuticals, and Takeda; speaking fees from AbbVie, Amgen, Ferring Pharmaceuticals, Shire, and Takeda; honoraria for advisory-board membership from AbbVie, Amgen, Boehringer Ingelheim, Celgene Corporation, Celltrion, Enterome, Ferring Pharmaceuticals, Genentech, Janssen Pharmaceuticals, Eli Lilly, Medimmune, Merck, Pfizer, Protagonist Therapeutics, Sandoz, Second Genome, Seres Therapeutics, Shire, Takeda, Theradiag, and Theravance Biopharma, and is a shareholder of Intestinal Biotech Development and Genfit. C.P. received consulting fees from Vitoria Laboratory, and travel support from Merck Sharp & Dohme and Abbvie. B.O. received research Grants from Dr. Falk Pharma, Takeda, Pfizer and Ferring; honoraria for advisory-board from Ferring, Pfizer, Janssen, Takeda, Abbvie, MSD, and speaker fees from MSD and Ferring. C.Y.P. received research grant and honoraria for advisory-board membership from Takeda and speaker's fees from Takeda, Abbvie Dr. Falk Pharma. J.M.J. received speaker fees from Janssen and advisory-board membership from MSD, Takeda, Janssen, Pfizer, Biogen, Fresenius, and Ferring. C.J.van der W. received research grants from Tramedico, Pfizer, and Takeda; speaker fees from Mundipharma, Celltrion, and Jansen and advisory-board membership from Pfizer, Takeda, MSD, Mundipharma, and Celltrion. J.T. received speaker fees from Takeda and consulting fees from Abbvie. The remaining authors disclose no conflicts.



Supplementary Figure 1. Kaplan–Meier time-to-event (aCRN) analysis for patients with IND; time from first IND within study interval to event ($P = .02$, log-rank test). aCRN was defined as CRC and/or HGD.

Supplementary Table 1. Inflammatory Parameters During Surveillance

	PSC-IBD	Non-PSC IBD	*P* value
Severity of active inflammation, mean (0–3)[a]	0.55	0.56	.89
Extent of active inflammation, mean (0–3)[a]	1.36	1.17	.003
Activity ratio for all surveillance colonoscopies, active:inactive	45%	41%	.19
No inflammation on all surveillance colonoscopies	76 (27.1%)	546 (34.1%)	.02
Inflammation extent, first colonoscopy			.001
No activity	127 (53.6%)	864 (57.9%)	
Limited	7 (3.0%)	89 (6.0%)	
Intermediate	38 (16.0%)	363 (24.3%)	
Extensive/pancolitis	65 (27.4%)	176 (11.8%)	
Inflammation extent, second colonoscopy			<.001
No activity	125 (55.3%)	866 (61.2%)	
Limited	9 (4.0%)	109 (7.7%)	
Intermediate	40 (17.7%)	297 (21.0%)	
Extensive/pancolitis	52 (23.0%)	141 (10.0%)	
Inflammation extent, third colonoscopy			<.001
No activity	102 (57.3%)	584 (63.6%)	
Limited	6 (3.4%)	79 (8.6%)	
Intermediate	22 (12.4%)	164 (17.9%)	
Extensive/pancolitis	48 (27.0%)	92 (9.9%)	
Endoscopic inflammation severity, first colonoscopy			.20
No activity	160 (57.1%)	924 (57.8%)	
Mild	100 (35.7%)	495 (30.9%)	
Moderate	19 (6.8%)	131 (8.2%)	
Severe	1 (0.4%)	50 (3.1%)	
Endoscopic inflammation severity, second colonoscopy			.77
No activity	125 (53.6%)	864 (59.3%)	
Mild	89 (38.2%)	445 (30.5%)	
Moderate	16 (6.9%)	101 (6.9%)	
Severe	3 (1.3%)	48 (3.3%)	
Endoscopic inflammation severity, third colonoscopy			.17
No activity	102 (55.4%)	583 (61.6%)	
Mild	63 (34.2%)	276 (29.2%)	
Moderate	13 (7.1%)	64 (6.8%)	
Severe	6 (3.3%)	23 (2.4%)	

[a]Corrected for total number of surveillance colonoscopies per patient.

Supplementary Table 2. Database Characteristics: Comparison The Netherlands and the United States

	The Netherlands (n = 1269)	United States (n = 642)	*P* value
Male	674 (53.1%)	327 (50.9%)	.37
Age at study inclusion, means (SD)	45 (12)	41 (15)	<.001
Age at IBD diagnosis, means (SD)	29 (12)	26 (14)	<.001
PSC	171 (13.5%)	122 (19.0%)	.002
Age at PSC diagnosis, means (SD)	33 (12)	32 (16)	.37
IBD type			<.001
Ulcerative colitis	800 (63.0%)	315 (49.1%)	
Crohn's colitis	434 (34.2%)	303 (47.2%)	
Indeterminate colitis	35 (2.8%)	24 (3.7%)	
Extensive disease/pancolitis	686 (54.1%)	338 (52.6%)	.56
Medication use			
Mesalamines	999 (78.7%)	543 (83.8%)	.008
Thiopurines	556 (43.8%)	362 (56.4%)	<.001
Biologicals	156 (12.3%)	284 (44.2%)	<.001
Number of surveillance colonoscopies, means	3.4	3.3	.25
Interval between surveillance colonoscopies, *y*, means	1.6	1.2	<.001
Neoplasia outcomes			
CRC	17 (1.3%)	12 (1.9%)	.37
HGD	15 (1.2%)	20 (3.1%)	.003
LGD	264 (20.8%)	88 (13.7%)	<.001

Supplementary Table 3. Univariate and Multivariate Cox-Regression Analysis for the Overall Risk of aCRN (All Patients), Corrected for Study Site

	Univariate			Multivariate		
Variable	HR	95% CI	*P* value	aHR	95% CI	*P* value
PSC	2.13	1.22–3.70	.008	1.85	1.00–3.43	.049
Inflammation severity (0–3)[a]	2.14	1.48–3.09	<.001	2.08	1.42–3.07	<.001
Sex (reference: male)	1.83	1.08–3.08	.02	1.68	0.97–2.89	.06
IBD type (reference: UC)	0.99	0.60–1.61	.95			
Maximum disease extent (reference: pancolitis)	1.43	0.85–2.41	.18			
Age at IBD diagnosis	1.00	0.98–1.02	.78			
Age (years)	1.02	1.01–1.04	.03	1.03	1.01–1.05	.004
Thiopurine exposure	0.84	0.51–1.40	.85			
Biological exposure	0.72	0.36–1.46	.36			
5-aminosalicylate exposure	1.14	0.58–2.25	.70			
Number of surveillance procedures	0.96	0.84–1.09	.53			
Population (reference: US)	2.82	1.72–4.62	<.001	2.20	1.30–3.74	.003

NOTE. aCRN was defined as colorectal cancer and/or HGD.
[a]Entered as time-changing covariate; 0, no inflammation/remission; 1, mild; 2, moderate; 3, severe.

Supplementary Table 4. Univariate and Multivariate Cox-Regression Analysis for the Risk of aCRN After Detection of LGD

	Univariate			Multivariate		
Variable	HR	95% CI	*P* value	aHR	95% CI	*P* value
PSC	2.52	1.19–5.31	.02	1.79	0.83–3.88	.14
Sex, reference: male	1.23	0.60–2.49	.57			
Thiopurine exposure	1.20	0.60–2.40	.60			
Biological exposure	0.74	0.23–2.44	.74			
Mesalamine exposure	1.07	0.44–2.59	.88			
Dysplasia characteristics						
Distal location	1.69	0.77–4.32	.17			
Multifocality	2.46	1.22–4.95	.01	1.90	0.93–3.87	.08
Polypoid morphology	0.27	0.13–0.57	.001	0.31	0.14–0.65	.002
Invisible dysplasia	1.64	0.76–3.53	.21			
Nonpolypoid morphology	1.82	0.70–4.74	.22			

NOTE. aCRN was defined as colorectal cancer and/or HGD.